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Preliminary ageing study of bioactive glass in a cell culture model

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In the past we sometimes found poor cell morphology and relatively low biochemical values for osteoblast cultures on bioactive glass. These observations were not caused by any external causes. Our hypothesis is that the surface reactivity of polished bioactive glass slides might decrease slowly due to the influence of (air-)humidity during storage under normal room conditions. In the present study we investigated the ageing of bioactive glass stored under room conditions as well as bioactive glass stored under dry conditions. We also compared the results with glass slides stored for about one year with freshly obtained bioactive glass slides, both stored under dry conditions. We evaluated several histological and biochemical parameters obtained from osteoblast cultures on the differently preserved glass slides. The results showed that bioactive glass probably maintains its bioactive nature when stored under dry conditions. We found different biochemical values for bioactive glass prepared with a new oven and elaborated with new polishing techniques in comparison with previously prepared batches of bioactive glass. These results indicate that different bioactive glass batches with identical composition may not be identical in terms of bioactivity.

1. Introduction

Bioactive glasses have excellent biocompatibility, and intimate bone bonding has been reported [1, 2]. These materials rapidly elicit a phenomenological corrosion behaviour after exposure in a physiological environment [3, 4]. A preferential leakage of sodium ions from the glass surface finally results in the formation of a calciumphosphate silica-rich top layer [1–4] which is supposed to be responsible for the bioactive nature of the glass after implantation in bone tissue [1, 2].

Our research so far has mainly concerned *in vitro* studies of osteoblasts cultured on several substrates, such as bioactive glasses, hydroxylapatite, titanium and quartz glass [5, 6]. In osteoblast cultures, histological and biochemical parameters were evaluated and it was concluded that these parameters were significantly better for bioactive glass than for the other materials mentioned above. We sometimes found poor cell morphology and decreased biochemical parameter values for bioactive glass. Infected cultures or certain errors during sample preparation or irrelevant biochemical measurements were excluded as possible causes. An internal cause could be a decrease in surface reactivity of the bioactive glass. Our hypothesis is that the highly charged surface of polished bioactive glass slides reacts slowly in contact with (air-)humidity during storage under normal room conditions.

The purpose of this study was to investigate the ageing of bioactive glass stored under room conditions as well as bioactive glass stored under dry conditions. We also compared bioactive glass stored for longer periods of about one year with bioactive glass obtained from a new batch. Both materials were stored under dry conditions. We used an established cell culture system [5, 6]. Osteoblasts were obtained from the calvariae of 20-day-old fetal rats and cultured for different periods of time. We evaluated the histological and biochemical parameters obtained from these osteoblast cultures [5, 6].

2. Materials and methods

The bioactive glass 45S5 according to Hench and coworkers [1] was prepared from high grade chemicals as described in our previous work [5, 6]. The prepared glass rods were sawn transversely and the slides obtained were ground and polished under water-free conditions (8 mm diameter, $\pm$ 0.4 mm thickness).

Polished glass slides were stored under room conditions in a container for longer periods of time to investigate the ageing of bioactive glass in terms of the cell culture model used. Time of storage is represented by X (Table I). These samples are termed type A.

Other glass slides of type A were stored under dry conditions in inert plastic containers in vacuum in a desiccator with a powerful drying agent. The glass slides were investigated in our cell culture model after 1–2 months. The remaining slides were preserved under dry conditions for longer periods of time (X = 12–14 months). Results are presented in Table II.

Polished glass samples were obtained from another batch and also stored under dry conditions. We used a new oven for the preparation and the slides were

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polished in the same manner as for glass A except that one part of the equipment was replaced by a new one from new acrylate. All other conditions remained unchanged during preparation and elaboration. These samples are designated as type B. The results are presented in Table III.

In every experiment some quartz glass slides served as a control. The cell culture growth could be followed with routine transmitted light microscopy. Histological observations and biochemical determinations were simultaneously carried out with bioactive glass samples.

The samples were sterilized by dry heat (150 °C) for 3 h before culture.

2.1. Cell culture method

Osteoblasts were isolated according to the methods described by Wong and Cohn [7] with some modifications [5, 6]. Calvariae from 20-day-old fetal rats (n = 10–15) were excized under aseptic conditions. The endo- and extracranial periostea were mechanically removed. The calvariae were incubated for 2 × 10 min at 37 °C with 4 mM EDTA in PBS. After rinsing the calvariae for 3 × 5 min with PBS, they were incubated for 10 min with collagenase (1 mg/ml PBS) at 37 °C. The cell suspension obtained was discarded as it contained periosteal fibroblasts, still present after removal of the periostea.

Osteoblasts were isolated by further collagenase treatment (2 × 30 min). The supernatant was centrifuged for 5 min at 1400 rpm (275 g). The pellet obtained was resuspended in culture medium, minimal essential medium (α-MEM), containing 5% inactivated fetal calf serum (FCS, Gibco), 1 mg/ml glucose and 90 μg/ml gentamycin. The sterilized glass samples were placed in 24-well culture dishes.

Samples for morphological studies were seeded with a cell density of 5×10^5 cells/ml. Samples for biochemical studies were seeded with a higher cell density (1×10^6 cells/ml) to obtain a well-defined signal with the spectroscopical techniques used, as established from pilot studies. On every glass sample a 50 μl cell suspension was applied with great care, to avoid unwanted cell attachment to the surrounding surface of the well, and the cells were allowed to attach for 2 h to the underlying glass substrate, then 750 μl of culture medium was also added carefully. During culturing the multi-well dishes were kept in an incubator (with 95% air humidity and 5% CO_2) at 37 °C. After 3, 6, 8 and 10 days half of the medium was replaced.

2.2. Histology

2.2.1. Scanning electron microscopy

The cultures were fixed after 2 and 12 days with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.4) for 2 h. The cultures were rinsed with PBS (3 × 5 min) and dehydrated in a graded ethanol series.

Prior to examination in a Philips SEM 525 M at 15 kV, the prepared samples were critical point dried and gold sputtered (Balzer).

2.2.2. Fluorescence microscopy

These cultures were fixed with 1% paraformaldehyde in cacodylate buffer. The osteoblast expression and its distribution over the monolayers was observed immunocytochemically after 12 days. The first monoclonal antibody (E 11, directed against a membrane-associated antigen of rat osteoblasts) was located with a goat-anti-mouse/FITC conjugate (Sigma). Observation of the samples was made with an Olympus BHRFL fluorescence microscope.

2.3. Biochemistry

2.3.1. Alkaline phosphatase activity and DNA-content

For both determinations [8, 9] we used the same culture. The ratio R was calculated from the determined values for the alkaline phosphatase activity (APA is a marker for osteoblast expression) and the corresponding DNA-content and the mean osteoblast expression (APA/per cell). Based on our earlier work [5, 6], we chose to determine both APA and DNA at the most relevant time interval (T = 8 days), as only the same sort of material was involved. The cultures were rinsed with PBS and stored at − 20 °C until use. Both determinations are described briefly below.

1. *APA* [8]. The samples were then placed in 1 ml PBS containing 0.05% Triton X-100 and sonified. To 10 μl of the sonified solution we added another 100 μl PBS/Triton followed by 100 μl (1 mM p-nitrophenylphosphate in 1 M diethanolamine + 1 mM $MgCl_2$, pH = 9.8). The mixture was incubated for 15–30 min at 37 °C until the colour was comparable with a standardized series (20 mM p-nitrophenol). With the addition of 1 ml 0.1 M NaOH the reaction was stopped and the samples were measured after calibration with the standard, on a Gilford N-300 photospectrometer at 410 nm.

2. *DNA* [9]. To the remaining solution of trypsin/tyrode buffer was added (with final trypsin concentration of 1 mg/ml) and the mixture was sonified again. To 0.5 ml of the solution 1 ml heparin solution was added (thromboliquine, Organon 5000 IU, 1:600, PBS) and 0.5 ml RNase (0.05 mg/ml PBS) (ribonuclease A, Sigma, USA). The mixture was allowed to react at room temperature for 0.5–1.0 h, and after addition of 0.5 ml ethidiumbromide (0.025 mg/ml PBS), the samples together with a standard were measured on a Perkin–Elmer LS-3B flourospectrometer at 590 nm.

2.3.2. Historical staining of APA

To visualize the distribution of the APA in the cultures histochemical staining [10] was used after 8 days. This method uses naphtholphosphate (MX, MS, Sigma, USA) as the reacting substrate. The cultures stained were observed at low magnification.

3. Results

3.1. Histology

3.1.1. Scanning electron microscopy

Fig. 1 illustrates the individual morphology of osteo-

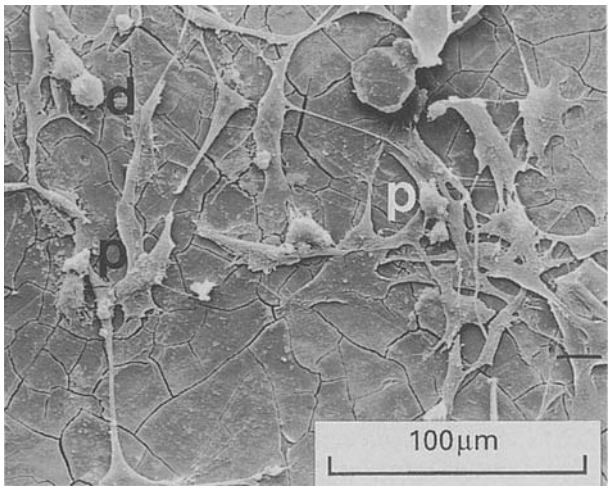


Figure 1 Scanning electron microscopical images showing osteoblasts cultured on aged bioactive glass. It can be seen that the morphology of individual cells (p) as well as the surface layer are very poor on aged bioactive glass. Sometimes, dead cells (d) are seen.

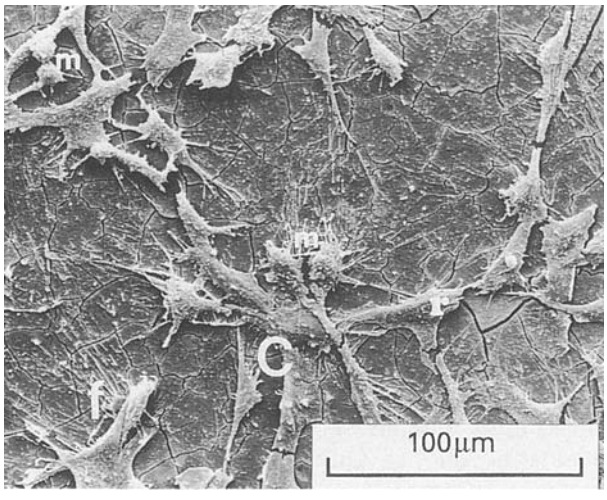
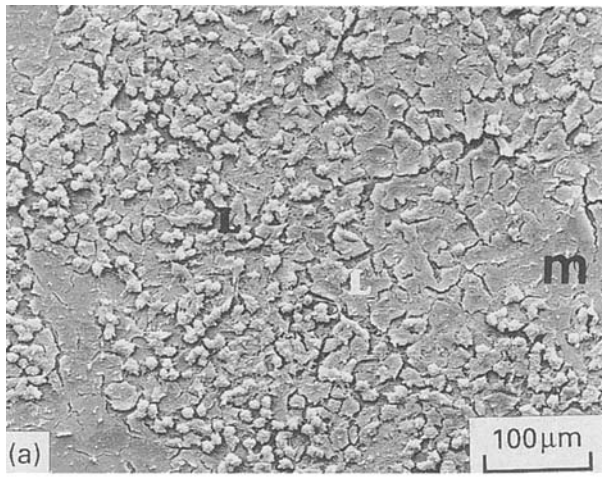


Figure 2 SEM micrograph of osteoblasts cultured for 2 days on bioactive glass stored under dry conditions (type A and type B). The osteoblasts mainly exhibit a "stand-off" morphology (C) with many dorsal ruffles (r) and filapodia (f). Also, cell divisions (m) are relatively often seen. The corroded layer (arrows) also shows precipitation of proteins (arrow).

blasts cultured for 2 days on aged bioactive glass ($X = 8$–10 months). The morphology of the cells was very poor and even dead cells were observed. The osteoblasts showed almost no dorsal ruffles. The surface layer lacked a regular precipitation and/or incorporation of protein.

In contrast, we found a good morphology of osteoblasts cultured for 2 days on bioactive glass slides which were stored under dry conditions (Fig. 2). The osteoblasts and surface layer showed a comparable morphology for bioactive glass types A and B. At the periphery we observed compact osteoblasts with a so-called "stand-off" morphology with relatively high dorsal activity [11]. At the centre the cells were more spread, in more close contact with each other and likely to form a monolayer. The cracks at the glass surface are due to dehydration of the top layer during



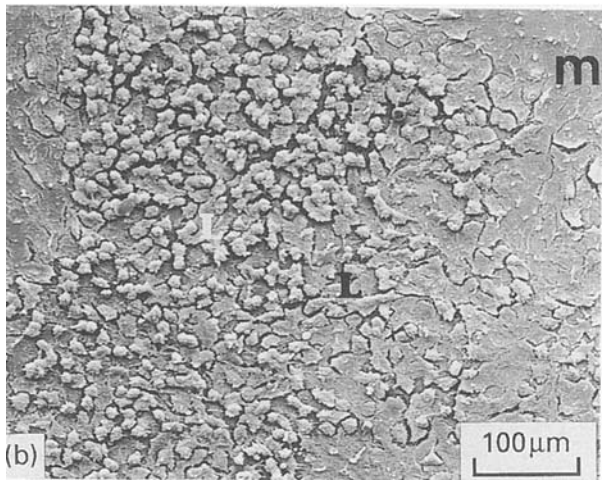


Figure 3 SEMs showing the morphology of osteoblasts on bioactive glass Type A (a) and type B (b) after 12 days culture. On top of the confluence monolayer (M) large clusters of cells (L) are seen. The clustered cells are mainly polygonally shaped, although some spherical cells can be detected.

critical point drying. This indicates the formation of a calcium-phosphate silica-rich top layer [4, 5]. We also observed precipitation and/or incorporation of serum-proteins on top of the surface.

Fig. 3 presents SEM-images of osteoblasts cultured for 12 days on bioactive glass types A and B which were stored under dry conditions. We observed large clusters and a confluent monolayer. The clustered cells were mainly polygonally shaped with strong dorsal activity, but spherical cells with blebs were also observed.

3.1.2. Fluorescence microscopy

On bioactive glass types A and B the osteoblast cultures showed a mild phenotypic expression over the monolayer. In the clusters the osteoblast expression was clearly higher. Most of the clustered cells were polygonally shaped, although spherical cells were also observed.

3.2. Biochemistry

3.2.1. APA, DNA and R

Table I presents the biochemical values (APA, DNA

TABLE I APA, DNA and R (= APA/DNA) values of osteoblast cultures (T = 8 days) on bioactive glass type A versus the time X of normal storage under room conditions

Number of sampes	APA (sd)	DNA (sd)	R (sd)	X (months)
1. 8	259.3 (20.6)	7.25 (1.14)	35.1 (3.2)	3
2. 12	293.4 (39.9)	7.01 (0.84)	42.0 (1.5)	4
3. 12	144.8 (10.8)	5.75 (0.19)	25.1 (1.5)	8
4. 6	175.3 (25.8)	7.32 (0.32)	23.9 (3.6)	10

TABLE II APA, DNA and R (= APA/DNA) values of osteoblast cultures (T = 8 days) on bioactive glass type A versus the time X of normal storage under dry conditions in a desiccator

Number of sampes	APA (sd)	DNA (sd)	R (sd)	X (months)
1. 8	273.7 (20.0)	8.53 (0.35)	32.2 (1.8)	1
2. 8	253.9 (67.5)	7.81 (1.03)	32.1 (4.5)	2
3. 22	250.7 (45.8)	7.09 (0.66)	35.5 (6.7)	13
4. 12	228.9 (29.6)	6.52 (0.52)	35.2 (4.3)	14

TABLE III APA, DNA and R values of osteoblast cultures (T = 8 days) on bioactive glass type B prepared with a new oven and elaborated with different polishing techniques

Number of sampes	APA (sd)	DNA (sd)	R (sd)	X (months)
1. 22	186.4 (46.1)	6.94 (0.56)	26.6 (4.8)	1
2. 12	161.6 (31.7)	7.10 (0.50)	22.7 (3.9)	2

and R) of osteoblast cultures (T = 8 days) on bioactive glass stored under room conditions. The results show that when bioactive glass was used within the first months of normal storage, high values were obtained (in comparison with other substrates, such as hydroxylapatite, titanium alloy and stainless steel). For longer periods of storage under room conditions the effect of the so-called ageing became manifest, and relatively low values for APA and R were found.

Table II presents the biochemical data of cultures (T = 8 days) on bioactive glass stored under special conditions. It can be seen that the biochemical values for well preserved glass slides did not remarkably change for periods from X = 1–2 to 12–14 months.

Table III presents the biochemical values of cultures (T = 8 days) obtained from bioactive glass prepared with a new oven and elaborated with polishing equipment where one part was replaced by a new one. The APA and R are lower for type B bioactive glass in comparison with glass type A (after X = 1–2 months preservation and even after storage for periods up to X = 12–14 months).

3.2.2. APA-staining

Fig. 4 presents the micrographs obtained from the histochemical APA-staining after 8 days. On both bioactive glass types A and B we observed confluent

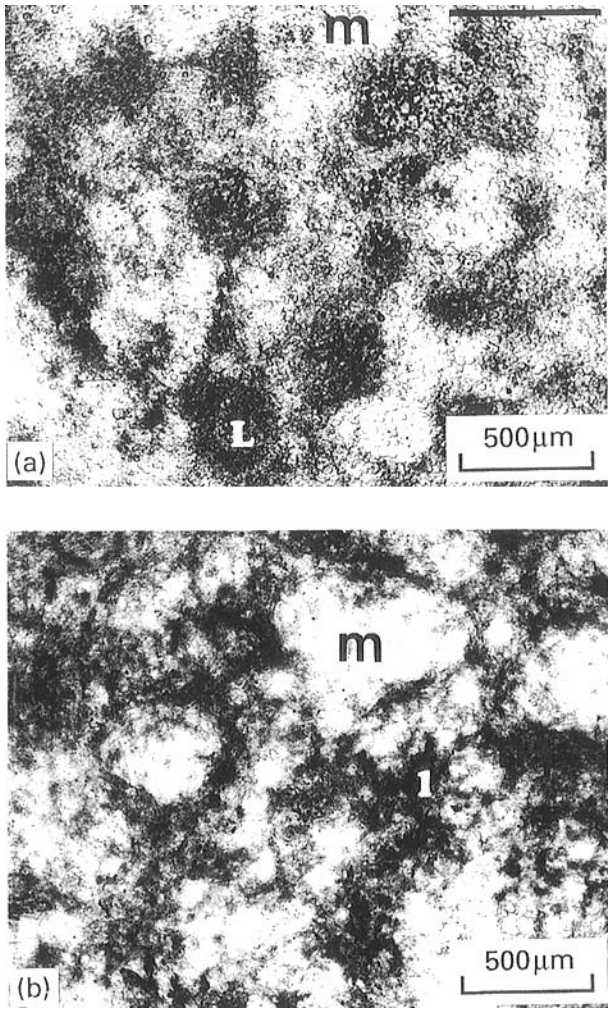


Figure 4 Light microscopical images of the histochemical staining for the APA after 8 days culture for type A (a) and type B (b) bioactive glass. Higher cluster (L) are seen over a confluent monolayer (m) on type A bioactive glass, whereas on bioactive glass type B the clusters are somewhat smaller (l). The enzyme activity on type A glass is higher than on type B glass and corresponds with our biochemical results.

cultures with a moderate enzyme activity over the monolayer, but higher activity in the clusters. For bioactive glass type A cultures we observed many large clusters, but for glass type B the clusters were on the average smaller (Fig. 4a and 4b).

4. Discussion

It is now well accepted that bioactive glasses have excellent biocompatibility [1, 2]. The biomechnical properties are indeed very poor, but in combination with a loadbearing device [12] the system delivers attractive bioactivity and durability. In contact with physiological fluids bioactive glass elicits very rapidly a complex of characteristic processes that result in the formation of a calcium-phosphate silica-rich top layer [3, 4]. To maintain this characteristic surface reactivity, bioactive glass needs to be kept away from water-based liquids until use.

Normally, good osteoblast morphology and high values for the biochemical parameters (APA, DNA-content and R) were found for bioactive glass in

comparison with other substrates, such as quartz glass, hydroxylapatite, titanium alloy and stainless steel as reported in earlier work [5, 6].

In the past we sometimes found poor histology and decreased values for the biochemical parameters of osteoblast cultures on bioactive glass. We observed a poor morphology for the osteoblasts and top layer of the glass surface as was shown in Fig. 1. At that time, special precautions for the preservation of the polished substrates were not taken: the samples were stored in a container under normal room conditions.

In an attempt to explain the observed decrease of parameter values we evaluated many possible causes. For example, the homogenicity of an osteoblast-enriched cell suspension [5–7] might vary slightly and when the amount of pre-osteoblasts dominates, the proliferation rate will be faster at the expense of phenotypic expression for these short culture periods. Also, the viability of survived cells brought into culture after the isolation procedure cannot be predicted with a trypan blue exclusion test. Another assumption was that uncontrolled action during critical point drying might result in poor cell morphology on bioactive glass, but then other simultaneously treated materials have to show this poor morphology too. Other causes such as errors during biochemical determination or yeast/bacterial infections were also excluded from our observations. We assumed that an internal cause was a decrease in surface reactivity of the bioactive glass.

In the present study we investigated bioactive glass versus time of storage under normal room conditions. The differences between the SEM images of Figs 1 and 2 were quite obvious with regard to cellular morphology and aspect of surface layer. These observations formed a strong indication for the presence of ageing on bioactive glass after longer storage time. The remarkably lower values found for the biochemical parameters after longer periods of time were also ascribed to the presence of ageing. These results were presented in Table I. We concluded that an ageing process was responsible for the decreased values of the osteoblast cultures of bioactive glass slides stored under room conditions for longer periods of time.

We also investigated bioactive glass slides stored under dry conditions for longer periods of time. We found good morphology of osteoblasts cultured for 2 days on well preserved bioactive glass. These observations were in agreement with the morphological observations described in earlier studies [5, 6]. Normally, osteoblasts cultured on bioactive glass are compact and have dorsal ruffles and filapodia. Cell divisions are relatively often seen (Fig. 2). The bioactive glass surface shows cracks due to dehydration after critical point drying and consists of a calcium-phosphate silica-rich top layer as was previously shown by microprobe analysis [5]. Precipitation and/or incorporation of proteins are also observed. From our observations and the biochemical data given in Table II it was shown that the ageing of bioactive glass can be prevented when the polished glass slides are preserved well.

In conclusion, when polished bioactive glass is stored under good (dry) conditions it will keep its surface reactivity for a considerably longer time than when stored under room conditions. Therefore, we found evidence that bioactive glass with its high free surface charge reacts slowly with the (air-)humidity, thereby losing some of its bioactive nature. We also compared bioactive glass type A stored for about one year with bioactive glass type B obtained from a freshly prepared batch. Both materials were stored under the same special (dry) conditions. We evaluated the histological and biochemical parameters obtained from osteoblast cultures on bioactive glass type A and type B. Osteoblasts cultured for 2 days on type B glass exhibited a comparable morphology to type A glass slides (Fig. 2). Confluent cultures on bioactive glass type A and type B substrata after 12 days of culture also showed comparable SEM and FM observations as described in the results (Fig. 3). These cultures showed large multi-layered cell clusters over a confluent monolayer, in which mainly polygonal cells with a high phenotypic expression were observed. This is in agreement with earlier observations [5, 6].

We found regular biochemical values of osteoblast cultures on bioactive glass type A which did not significantly change during the period of storage under dry conditions (Table II). Surprisingly, the APA and R values were lower for bioactive glass type B (Table II).

The APA-staining in this study visualized the distribution of the enzyme activity in the osteoblast cultures after 8 days and corresponds with the above-mentioned biochemical determinations. It was shown that the APA is higher on bioactive glass type A than on bioactive glass type B (Fig. 4a and 4b).

In previous reports we stated that the glass-preparing method was reliable and reproducible [5]. However, the preparation for the type B batch of bioactive glass was carried out with new equipment, one of the polishing parts being replaced by a new one made from new acrylate, and remnants of acrylate monomer might have stuck to the glass slides during polishing. This might be the cause of the lower APA in the cells cultured upon glass slides from this batch. From our own cell culture experience we know that acrylate monomers are detrimental to cell cultures. Therefore, we state that constant conditions for equipment and elaboration are of great importance in maintaining reproducibility during the preparation of different batches of bioactive glass with the same culture characteristics.

Further research for the improvement of preparation and storage of bioactive glass is recommended, especially when bioactive glass is to be applied in clinical studies [12].

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References

1. L. L. HENCH, R. J. SPLINTER, W. C. ALLEN and T. K. GREENLEE, *J Biomed. Mater. Res. Symp.* **2** (1971) 117.
2. L. L. HENCH and H. A. PASCHALL, *ibid.* **4** (1973) 25.
3. D. M. SANDERS and L. L. HENCH, *J. Amer. Cer. Soc.* **56** (1973) 373.
4. L. L. HENCH, *J. Non-Cryst. Solids* **19** (1973) 27.
5. W. C. A. VROUWENVELDER, C. G. GROOT and K. DE GROOT, *Biomaterials* **13** (1992) 382.
6. *Idem., J. Biomed. Mater. Res.* **27** (1993) 465.
7. D. V. COHN and G. L. WONG, in "Skeletal research" (Simmons & Kunin, NY, 1979) p. 3.
8. U. KARSTEN and A. WOLLENBERGER, *Anal. Biochem.* **77** (1977) 464.
9. O. A. BESSEY, O. H. LOWRY and M. J. BRECK, *J. Biol. Chem.* **164** (1946) 321.
10. M. S. BURNSTONE, *J. National. Cancer Inst.* **20** (1958) 601.
11. J. E. DAVIES, B. CAUSTON, Y. BOVELL, K. DAVY and C. S. STURT, *Biomaterials* **7** (1986) 231.
12. P. DUCHEYNE, *J. Biomed. Mater. Res.* **19** (1985) 273.

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