JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 13 (2002) 549–556

The effect of MgO on the solubility behavior and cell proliferation in a quaternary soluble phosphate based glass system

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This paper presents a systematic study of the MgO–CaO–Na_2O–P_2O_5 glass system, which has great potential to be used as temporary hard and soft tissue implant materials. An overall study of solubility behavior of ternary and quaternary-based phosphate glass system have been carried out in order to understand the out-leaching progress of different ions and to determine their effect on cell proliferation. Originally, soluble phosphate based glasses within the ternary glass system of Na_2O–CaO–P_2O_5 have been developed to create a simple baseline system. This paper, however, presents the development of this system by introducing magnesium oxide as a partial calcium oxide substitute and solubility behaviors as well as cell studies have been carried out to check the effect on magnesium ions.

Glasses have been prepared via standard glass melting techniques and their solubility behavior has been tested in distilled water via simple weight loss, pH and ion measurements. The way the glasses dissolve is an inverse exponential behavior which is mirrored by the calcium ion release. Other ions show a less exponential behavior. The MTT test has been used to check preliminary *in vitro* studies on a human MG63 cell line and the result indicates that cell proliferation is increased for glasses with minimal CaO substitution.

Introduction

Biomaterials research has developed a number of materials for hard and soft tissue replacement and augmentation, such as synthetic hydroxyapatite (HA) [1–6] Bioglass® and bioactive glasses [7–11] or apatite based glass–ceramics [11–16]. There are two major positive features occurring:

1. A stable attachment is formed between implant and bone, which reduces the risk of implant loosening with subsequent loss.
2. The chemical relationship between the implant and surrounding tissue is not dissimilar, thus reducing the effects of corrosion, rejection, allergic or inflammatory reactions.

However, the above-mentioned materials often involve the use of silica, the long-term effect of which is not known. Nagase *et al*. [17] examined the toxic effect on mice when suspensions of calcium phosphate glasses containing silica were injected. Calcium phosphate glasses without silica showed no toxicity. The silica containing suspension however indicated toxicity [17]. This study presents an alternative material, which is not only chemically related to the natural phase of bone but is also easy to make and cast in any required shape. Furthermore, it offers the characteristic of being soluble within a wide spectrum of solubilities and when used as a temporary device, would not necessitate of a second device removal operation. This material could act as a replacement of PTFE membranes and other non-degradable temporary polymeric devices which are currently used for periodontal treatment [18–25] as well as other periodontal and maxillofacial procedures and require removal after they have performed their function.

This study showed that it is possible to create soluble phosphate-based glasses with a range of solubilities from highly soluble (2–3 h) to relatively stable glasses (one year and possibly longer).

A relatively simple ternary phosphate based glass system was investigated and is described elsewhere [26]. On the basis of this system, MgO was introduced to partially replace CaO as a network modifier and measure

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the effect on the solubility behavior and the cell proliferation. The knowledge of how the cells will react towards an implant is of great importance since it is a crucial prerequisite for any development of novel biomaterials. From such results, tissue-biomaterial interactions can be derived and will help to develop better materials.

Materials and methods

Glass preparation

NaH_2PO_4, $CaCO_3$, $MgCO_3$ and P_2O_5 precursors were used for this glass system, which were obtained from BDH, England. Glasses were prepared via glass melting techniques, detailed elsewhere [26]. Chemical compositions were obtained via previous calculation methodology again detailed elsewhere [26]. For melting temperatures and casting temperatures see Table I. The glass compositions were made such that the P_2O_5 content was kept fixed as was the Na_2O content and the divalent oxide ration, i.e. the CaO : MgO ratio was varied (see Table I). After casting the glasses into 1.5 cm diameter by approximately 6 cm long rods, the rods were cut into 1.5 mm thick disks on a Testbourne diamond circular saw. After cutting, disks were stored in sterile test tubes (Sterilin, England).

Solubility test

The surface area of the glass disks was calculated from measurements of thickness and diameter, measured with vernier callipers (Mitutoyo, Tokyo, Japan). The samples were then weighed on an analytical balance (Precisa 120A, European Instruments) and the disks were placed in 75 ml plastic containers, which were filled with 25 ml double distilled water. The containers were sealed with plastic lids and stored over the whole testing period in an incubator at 37 °C. The maximum testing time was eight weeks. At various time points, the specimens were taken out, surface moisture dried with tissue and reweighed. They were then placed back into solution.

The weight loss M_t at a certain time t point was subtracted from the initial weight M_0 and divided by surface area A to give weight loss per unit area:

$$\text{Weight loss} = (M_0 - M_t/A)$$

The values were then plotted against time and from the resultant graph the solubility may be estimated by fitting a line to the data that goes through zero. This gives solubilities in $\text{mg cm}^{-2}\,\text{hr}^{-1}$. All measurements were performed in triplicate.

Ion measurements

pH and ion measurements were carried out at the same time as the weight loss measurement took place. The pH was measured with an Orion pH meter which was calibrated with colorkey standard 4, 7 and 10 obtained from BDH, England. The ion measurements were carried out on a Jenway 2240 Ion Meter using ELIT mono solid state ion selective electrodes with appropriate reference electrodes. Therefore, Ca^{2+}/AgCl and Na^{+}LiAc (Elit mono solid state electrode/corresponding reference electrode) were used. Calcium standard solution was obtained from Russell (Russell pH Limited, Scotland) and the sodium standard solution was obtained from Aldrich.

MTT tests

The MTT test assay (Chemicon, Temecula Ca., USA) has been useful for examining the effects of extracts from soluble glasses on the proliferation of a bone cell line. In this study the MTT assay was used to determine the cytotoxicity of magnesium glasses with different CaO/MgO ratios to determine the effect of magnesium.

The substance used is a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium salt, which turns into a blue formazan product due to the viable mitochondria in active cells. The product can be colorimetrically analyzed at 570 nm. Glass extracts were obtained by incubating glass disks in normal culture medium for 24 h. This ''conditioned'' medium was termed ''neat'' (N) and subsequent dilutions were made to give extracts of 1/4, 1/16 and 1/64 dilution. Extracts were aseptically filtered via 0.22 μm filters.

Cells of the human osteoblast cell line (MG63) were incubated in culture medium containing glass extracts for two days and five days and the cell proliferation was measured using the MTT test. Cells were plated at a density of 2000 cells/well in replicate 96 well culture plates and allowed to attach overnight at 37 °C, after which, the media were removed and replaced by diluted/neat extracts. Cells, maintained in normal culture medium, were used as controls. Absorbance at 570 nm was measured using a Titertek Multiskan Spectrophotometer (Labsystems, Helsinki, Finland).

The results were expressed as the average absorbance of six replicate wells and normalized with respect to the control cells grown on tissue culture plastic, by dividing the test values by the tissue culture control values. Hence, a value of one indicates proliferation similar to the tissue culture plastic, below 1 indicates reduced proliferation and above 1 an increased proliferation with respect to tissue culture plastic.

TABLE I Glass compositions, codes used, melting and casting temperatures

Glass code	CaO content (mol %)	MgO content (mol %)	Na_2O content (mol %)	P_2O_5 content (mol %)	Melting temperature and time (°C/Hours)	Casting temperatures (°C/Hours)
$Ca_{32}Mg_0Na_{23}P_{45}$	32	0	23	45	1200/3	350/1
$Ca_{30}Mg_2Na_{23}P_{45}$	30	2	23	45	1200/3	350/1
$Ca_{25}Mg_7Na_{23}P_{45}$	25	7	23	45	1200/3	350/1
$Ca_{20}Mg_{12}Na_{23}P_{45}$	20	12	23	45	1200/3	350/1
$Ca_{15}Mg_{17}Na_{23}P_{45}$	15	17	23	45	1200/3	350/1
$Ca_{10}Mg_{22}Na_{23}P_{45}$	10	22	23	45	1200/3	350/1

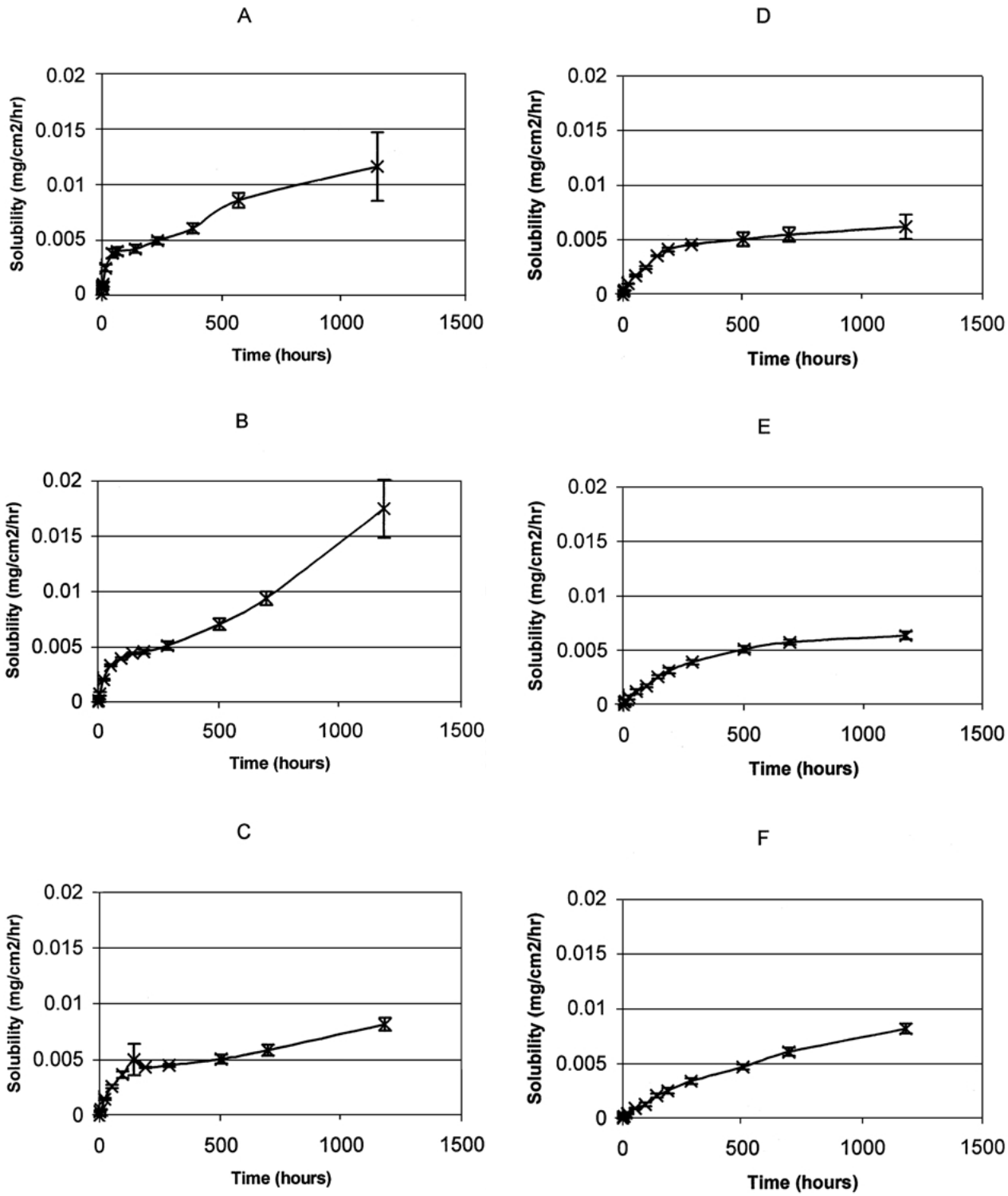


Figure 1(A)–(E) Weight loss per unit area against time for glasses $Ca_{32}Mg_0Na_{23}P_{45}$ (A), $Ca_{30}Mg_2Na_{23}P_{45}$ (B), $Ca_{25}Mg_7Na_{23}P_{45}$ (C), $Ca_{20}Mg_{12}Na_{23}P_{45}$ (D), $Ca_{15}Mg_{17}Na_{23}P_{45}$ (E) and $Ca_{10}Mg_{22}Na_{23}P_{45}$ (F).

Results

Glass preparation

Data is presented for six glasses. Attempts were made to increase the MgO content above 22 mol % but these were unsuccessful due to spontaneous crystallization of the glass.

Because of the relative ease with which crystallization occurred in the casting, the temperatures were carefully chosen. If the casting temperature was too close to the glass transition temperature (T_g) crystallization occurred. Thus to optimize the glass forming process, T_g and the crystallization temperature (T_c) were measured via differential thermal analysis. T_c was found to be around 500 °C and the casting temperature was chosen to be lower than this. The temperature of 350 °C was chosen as this gave non-crystalline materials with little residual stress.

Solubility

Previous work on a ternary Na_2O–CaO–P_2O_5 glass system showed, an inverse exponential relationship between weight loss per unit area and time and was found to describe the degradation process [26]. It was

TABLE II Solubility results: overview for the phosphate glasses

Glass code	Solubility [$g \cdot cm^{-2} \cdot h^{-1}$]
$Ca_{32}Mg_0Na_{23}P_{45}$	0.0122
$Ca_{30}Mg_2Na_{23}P_{45}$	0.015
$Ca_{25}Mg_7Na_{23}P_{45}$	0.0085
$Ca_{20}Mg_{12}Na_{23}P_{45}$	0.0072
$Ca_{15}Mg_{17}Na_{23}P_{45}$	0.0072
$Ca_{10}Mg_{22}Na_{23}P_{45}$	0.008

found that within the degradation process, the CaO content is the most influential factor controlling the degradation process: the more CaO is incorporated the lower the solubility and also the more non-linear the solubility curve becomes. In this study it was hypothesized that if MgO were used as a CaO substitute, the degradation process would be minimally influenced due to the similar valency of Ca^{2+} and Mg^{2+}.

Fig. 1(A)–(F) depict the effect of increasing MgO content and consequently decreasing CaO content. It can be seen that with increasing MgO content the degradation becomes more linear with time. Table II gives the solubility in $[mg\,cm^{-2}\,h^{-1}]$. It can be seen that the substitution of CaO with MgO in general decreases the solubility of the glass material. It should be noted that the lines fitted are in certain cases being fitted to nonlinear data and thus will be giving an approximate figure. This may also account for the deviation seen in the data from the general rule stated above.

PH measurements

For the following pH and ion measurements, data will be presented for glasses with a fixed P_2O_5 and Na_2O content and varying CaO : MgO ratio.

For clarity, the data has been split into two figures, with Fig. 2(A) showing the pH measurements for glasses with 0, 2 and 7% MgO content. The figure shows that initially there is a significant increase in pH from a starting value of around 5 for distilled water up to around neutral and then followed by a slow decline to around 6. There is some evidence that the glass containing 7 mol % MgO inhibits the drop in pH at long time periods. Fig. 2(B) shows the pH change with time for the glasses with 12, 17 and 22 mol % MgO. These show the same general trends but the highest values reached are only around 6.5 and then the pH tends to drop only a minimal amount with time for all three glasses. There is no significant difference between the three curves.

Ca^{2+} measurements

For the Ca^{2+} ion release measurements, for the glasses with 0, 2 and 7 mol % MgO (Fig. 3(A)), Ca^{2+} is initially released at a relatively high rate up to approximately 200 h. The release rate then slows down. For these three glasses there is little significant difference between the three curves. For the glasses with higher MgO content (12, 17 and 22 mol %, Fig. 3(B)) the Ca^{2+} ion release appears to show an initial lag, followed by a period of high rate of release which then after about 200–300 h,

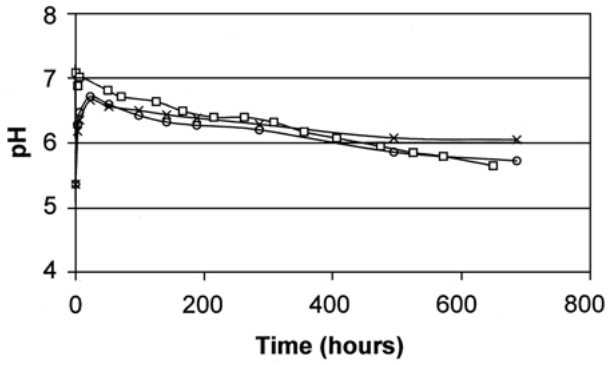


Figure 2(A) Change in pH with time for □ $Ca_{32}Mg_0Na_{23}P_{45}$ ○ $Ca_{30}Mg_2Na_{23}P_{45}$ and × $Ca_{25}Mg_7Na_{23}P_{45}$.

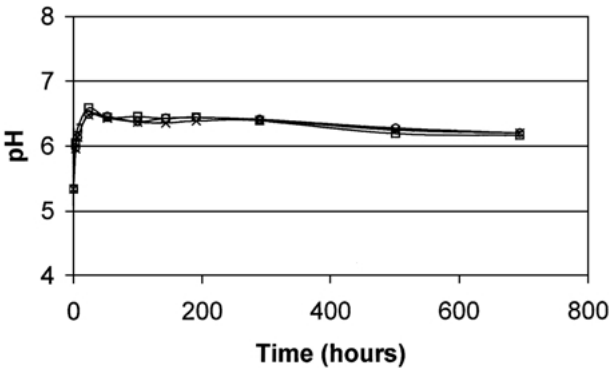


Figure 2(B) Change in pH with time for □ $Ca_{20}Mg_{12}Na_{23}P_{45}$ ○ $Ca_{15}Mg_{17}Na_{23}P_{45}$ and × $Ca_{10}Mg_{22}Na_{23}P_{45}$.

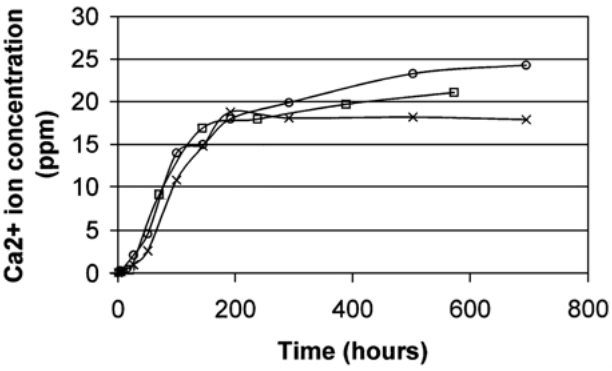


Figure 3(A) Change in Ca^{2+} ion concentration for □ $Ca_{32}Mg_0Na_{23}P_{45}$ ○ $Ca_{30}Mg_2Na_{23}P_{45}$ and × $Ca_{25}Mg_7Na_{23}P_{45}$.

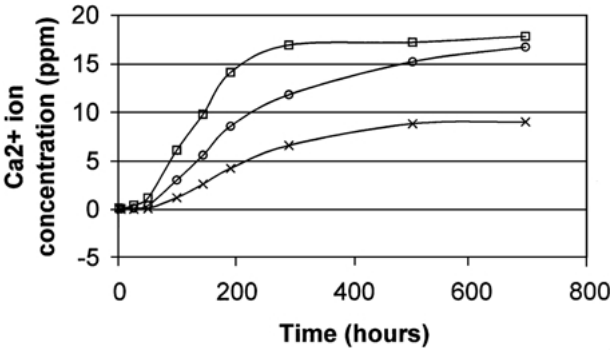


Figure 3(B) Change in Ca^{2+} ion concentration for □ $Ca_{20}Mg_{12}Na_{23}P_{45}$ ○ $Ca_{15}Mg_{17}Na_{23}P_{45}$ and × $Ca_{10}Mg_{22}Na_{23}P_{45}$.

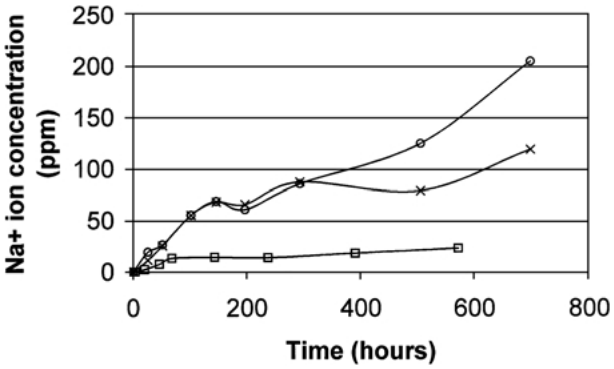


Figure 4(A) Change in Na^+ ion concentration for □ $Ca_{32}Mg_0Na_{23}P_{45}$ ○ $Ca_{30}Mg_2Na_{23}P_{45}$ and × $Ca_{25}Mg_7Na_{23}P_{45}$.

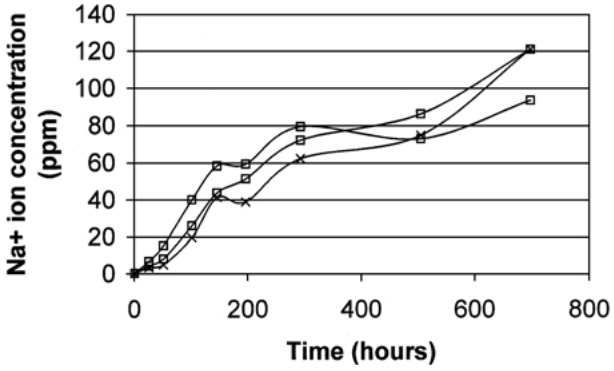


Figure 4(B) Na^+ ion measurement for $Ca_{20}Mg_{12}Na_{23}P_{45}$, □ $Ca_{15}Mg_{17}Na_{23}P_{45}$ and △ $Ca_{10}Mg_{22}Na_{23}P_{45}$.

slows down. There is also a correlation between MgO content and Ca^{2+} release. As the MgO content increases the solubility decreases.

Na^+ measurements

For the glasses with low MgO content (0, 2 and 7 mol % MgO, Fig. 4(A)), the glass with no MgO shows the lowest rate of Na^+ ion release. The other two glasses show similar rates of release up to about 300 h after which the curves diverge.

For the glasses with higher MgO content (12, 17 and 22 mol %, Fig. 4(B)) the curves all follow a similar rate of release and no significant differences may be seen between the three curves.

MTT tests

Fig. 5(A)–(F) show the results for the MTT test for the five glasses containing MgO and the control glass without MgO. The data show the effect of both glass composition and also dilution on cell proliferation. There is a difference in the growth of MG63 cells depending on the time in culture, concentration and composition of the extract used.

Cells exposed to the extracts without Mg showed a slightly decreased proliferation after two days compared with control cells in the presence of extracts 1/64 and 1/16 (Fig. 5(A)). However, the more concentrated extracts, 1/4 and ''neat'', showed an equal and enhanced cell proliferation, respectively, compared with control cells. This phenomenon was also evident for extracts containing 2 mol % MgO after two days in culture (Fig. 5(B)). After 5 days incubation, extracts containing no Mg did not affect cell growth compared with control cells (Fig. 5(A)). However, extracts containing 2 mol % Mg showed an increase cell proliferation with increasing extract concentration, thus there was a 15% increase in cell growth in the presence of the ''neat'' extract compared with control (Fig. 5(B)).

Glass extracts with 7% Mg, after two and five days in culture, showed cell proliferation equal to or slightly higher than control cells at all dilutions except for 'neat', which resulted in a decreased growth to 80% of control levels (Fig. 5(C)). Moreover, increasing Mg concentration to 12 mol % had little effect on cells compared with control cells after two days in culture (Fig. 5(D)). However, after five days, there was a clear increase in cell growth at all extract dilutions, up to 20% more than control cells in the presence of 1/64 and 1/16 dilutions, in particular (Fig. 5(D)).

In contrast, cells exposed to extracts containing higher concentrations of 17 mol % and 22 mol % exhibited very similar growth compared with control cells, except in the case of ''neat'' extract, which suggested a slightly enhanced proliferation, after two days (Fig. 5(E) and (F)). By contrast, after five days in culture, these glasses showed increased cell proliferation, up to 20% more than control cells, although no clear correlation between extract dilution and the amount of cell proliferation was evident (Fig. 5(E) and (F)).

These results suggest that, in the presence of a series of dilutions, the glasses containing 2–22 mol % Mg, show no obvious deleterious effects on the viability and proliferation of MG63 cells, even in the presence of ''neat'' extracts.

Discussion

Solubility

This glass system study was carried out by systematically replacing CaO with MgO to determine the effect of replacing one ion with another of the same valence, but with a differing ionic radius and to determine how this affected the solubility behavior. It was hypothesized that MgO will make no difference towards solubility behavior of the glass. It was demonstrated in earlier studies that the CaO content plays a major contribution in terms of solubility behavior of the glass. It can be seen from this study that by systematically replacing CaO with MgO, the solubility curves lose their exponential nature. This might be deduced as evidence that CaO makes a significant contribution in controlling the solubility process more than, for example, sodium oxide or potassium oxide, which in earlier studies were shown not to have a major effect. It is interesting that substituting a larger ion (Ca^{2+}) with a smaller ion (Mg^{2+}) reduces the solubility. It would be expected that the opposite would occur as the Mg^{2+} ion would be more mobile and diffuse more easily out of the structure but this is not the case. An explanation for this has not been found. However, the solubility values in Table I are calculated from a line fit to non-linear data and so may be giving unrepresentative values.

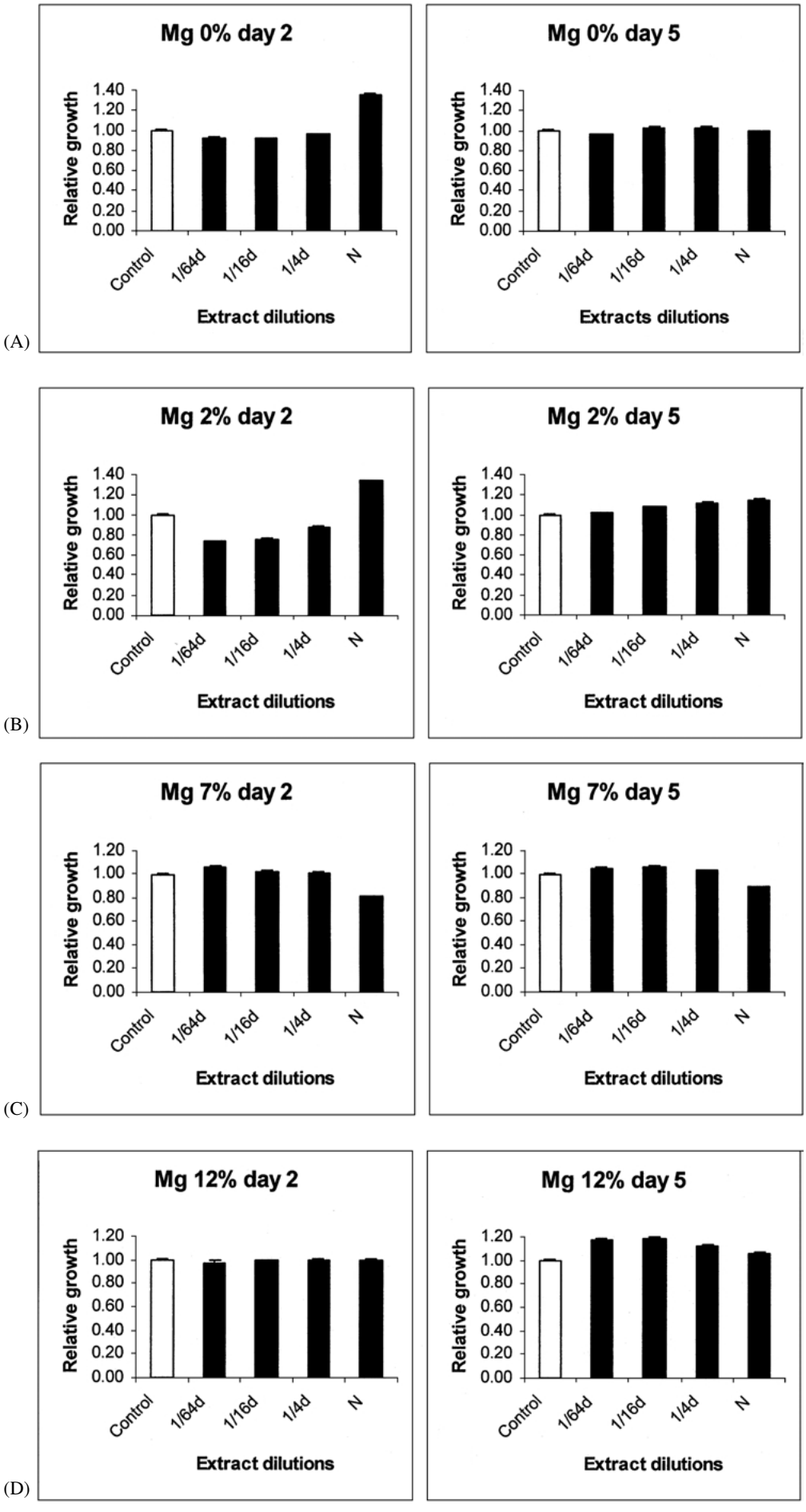
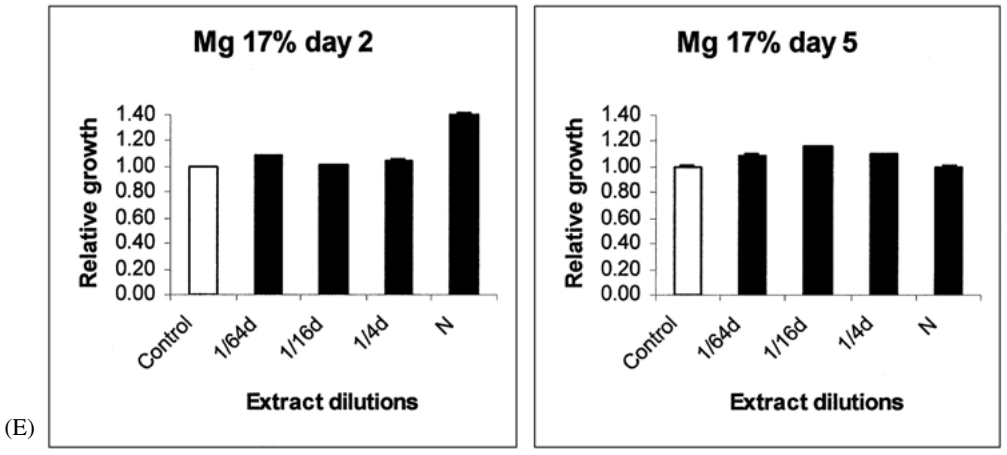
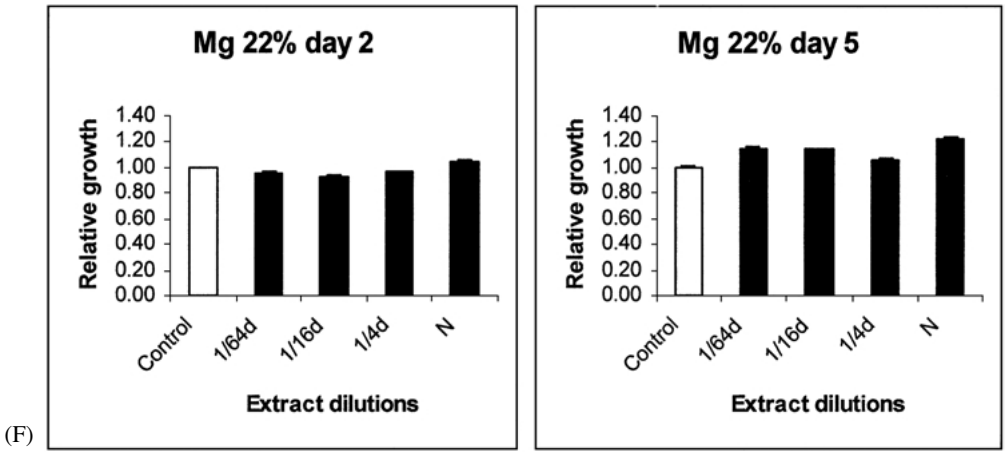


Figure 5 MTT assay of the effects of glass extracts on the proliferation of MG63 cells after two and five days of culture. White bars = control cells incubated with medium only and no glass extract; black bars = cells incubated in the test extracts. Vertical lines represent $\pm$ SD ($n = 6$). (Ratios of the mean absorbance levels (570 nm) of cells incubated with the extracts compared with the mean absorbance levels of the control cells were calculated and are shown as the relative growth.)



(E)



(F)

Figure 5 (Continued)

pH and ion measurements

The pH change with time was found to be similar to other systems. However, the glass with the highest MgO content showed the lowest decrease in pH even for longer time periods. Both ion measurements were found to be as expected and are very similar to other glass systems. Again, a lower CaO content exhibited a less exponential ion concentration curves, higher CaO containing glasses showed a more exponential figure, which is reflected in the weight loss curves.

MTT

In this study, we examined the proliferation of a human osteoblast-like cell line, MG63, cultured, up to 5 days, in the presence of extracts of a quarternary series of soluble phosphate glasses containing different amounts of Mg. The cell line has previously been used in biocompatibility studies because it exhibits a number of features, which are similar to normal human osteoblasts.

Analysis of the growth of the cells using the MTT assay compared with the control cells incubated in normal medium, indicated that there were some differences in the cell growth depending on the extract dilution and composition of the glass. In particular, the slight decrease in cell proliferation in extracts containing little or no MgO was only evident after two days in culture. By five days, however, extracts of all glass formulations typically showed growth levels equal to or better than growth seen in control cells.

Although the precise mechanisms which are responsible for the apparent beneficial effects of these glasses on MG63 cells are unclear, it seems that the development of a calcium phosphate-rich layer produced by the glasses on dissolution for 24 h in medium, may improve the biocompatibility and therefore augment any changes in cell proliferation in the presence of the resulting glass extracts.

Conclusion

By replacing CaO with MgO the solubility curve loses its exponential nature. This might be deduced as evidence that the CaO makes a significant contribution to controlling the solubility process. The pH change with time was found to be similar to the ternary Na_2O–CaO–P_2O_5 system [26], however, the glass with the highest MgO content shows the lower decrease in pH even at longer time periods. Both ion measurements were found to be similar to the ternary system in both concentration and release rate [26]. A lower CaO content showed a less exponential ion concentration curve, higher CaO containing glasses showed a more exponential figure, which mirrors the weight loss curves. In addition the MTT assay suggested the growth of MG63 cells in the presence of the glass extracts of four different dilutions remained largely unaffected, and after five days

in culture, apparently increased the cell proliferation in some cases, particularly for those glasses containing 7 mol % Mg or more.

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Received 9 May
and accepted 23 October 2001