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Dependence of *in vitro* biocompatibility of ionomeric cements on ion release

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The *in vitro* biocompatibility of a group of ionomeric cements (ICs) was evaluated with respect to their ion release properties. These ICs were made from a defined series of glasses with the general formula $1.5SiO_2 \cdot 0.5P_2O_5 \cdot Al_2O_3 \cdot (1.0\text{-}Z)CaO \cdot 0.75CaF_2$ where Z was the mole fraction (ranging from 0–0.1) of an alkali metal oxide, either sodium or potassium or a mixture of both. For these alkali metal ICs, the amount of sodium released was directly related to the sodium content of the constituent glass. Similarly, the amount of potassium released was directly related to the potassium content. There was no correlation between the aluminum content of the glass and the aluminum ion release. Increasing the monovalent cation concentration, however, produced ICs with increased fluoride release. The biocompatibility of the ICs, as assessed by *in vitro* cell growth and viability measurements, was inversely proportional to aluminum ion release. Fluoride ion release, although important in terms of *in vitro* biocompatibility, would appear to be less important than aluminum ion release in determining the overall biocompatibility of the ICs studied.

1. Introduction

Ionomeric cements (ICs) are based on the neutralization reaction of ion-leachable inorganic glass particles with an organic polyelectrolyte (polyacrylic) acid. Similar ionomeric materials are already successfully used for dental restorations because of their properties which include a rapid set, adhesion to enamel and dentine, and release of fluoride ions which are thought to confer resistance against dental caries [1]. Additional properties that make ionomeric materials attractive as bone substitutes include a non-exothermic setting reaction with no thermal damage to tissues at the implant bed, minimal shrinkage on gelation, chemical adhesion to mineralized tissues and metals, the possibility of incorporating drugs, and growth factors within the cements, and improved bioactivity due to the release of potentially biologically active ions [2–4]. These materials have been evaluated for use as orthopaedic cements and bone substitutes in oral and maxillofacial surgery, having advantages over acrylic cements and hydroxyapatite or tricalcium phosphate bone substitutes [3, 5].

The existing structural model suggests that ICs are essentially hybrid glass polymer composites, consisting of inorganic glass particles surrounded by a silicon-rich layer in an insoluble hydrogel matrix which is held together by a combination of ionic cross-links, hydrogen bridges and chain entanglements [1, 6, 7]. It has been suggested that exchange of ions with adjacent tissues at the implant site is the basis of the osteoconductive and bone-bonding properties associated with ICs [8–11]. X-ray microanalytical studies of set ICs have shown that ions released from the glass particles during the gelation process are present in the matrix of the cement [7] and in adjacent bone [8]. Even after gelation has occurred, there is mobility of ions within the IC and exchange of ions takes place with the (aqueous) environment [1, 12, 13]. It is also likely that certain ions have a direct and dose-dependent action on bone cells at the implant site. Low concentrations of aluminum and fluoride ions have been reported to stimulate the proliferation and differentiation of osteoblasts *in vitro* [14, 15], while *in vivo* aluminum and fluorine administration have been shown to increase bone volume [16–19]. Higher concentrations of aluminum and fluorine have been shown to be harmful to bone, for example aluminum-induced bone disease, and are undesirable [20–22]. The aim of this study was to investigate if ions released from ICs affect their *in vitro* biocompatibility. In particular, we were interested in the effects of aluminum and fluorine on cell growth and viability.

2. Materials and methods

2.1. Materials

The ICs evaluated were made from a defined series of glasses with the general formula $1.5SiO_2 \cdot 0.5P_2O_5 \cdot Al_2O_3 \cdot (1.0\text{-}Z)CaO \cdot 0.75CaF_2$ where Z was the mole fraction of an alkali metal oxide and where Z for ICs LG2 = 0, LG5 = 0.05Na, LG6 = 0.1Na, LG8 = 0.0525K and LG11 = 0.05Na/K. In contrast

to ionomer glasses currently used in commercial materials, and early ICs, these glasses were single-phase homogeneous glasses free of any crystalline inclusions or glass in glass phase separations. They were produced by the Department of Materials Science, University of Limerick, with compositions to give different ion release profiles, this effect being achieved by increasing the proportion of monovalent alkali metal in the glass.

2.2. Ion release

Discs of ICs (20 mm diameter, 2 mm thick) were produced by mixing glass powder, freeze-dried mercaptan-free polyacrylic acid (Advanced Healthcare, UK) and sterile non-pyrogenic water in the ratio 1 : 0.2 : 0.5. They were then allowed to undergo gelation for 24 h at 37 °C and 100% relative humidity prior to evaluation. Ions were eluted under sink conditions into sterile high-purity water at 37 °C, samples being collected at 1, 3 and 6 wk. Fluoride ion concentrations were determined using a calibrated ion selective electrode (Orion Research Incorporated, Cambridge, MA 02139, USA) with and without TISAB III buffer (Orion Research Incorporated) to determine total and free (unbound) fluoride concentrations, respectively. Aluminum, sodium and potassium ion determinations were carried out using atomic absorption spectroscopy. Statistical analysis was undertaken using the unpaired Student's *t*-test.

2.3. *In vitro* biocompatibility

For *in vitro* studies, bone marrow cells were aseptically removed from dissected rat femora, and cultured in supplemented MEM-alpha medium in 75 ml flasks at 37 °C in an atmosphere of 5% CO_2 [23]. The MEM-alpha medium was supplemented with 15% heat-inactivated foetal calf serum, 10 mM beta-glycerophosphate, 50 $\mu g\,ml^{-1}$ ascorbic acid, 10 nM dexamethasone, 50 $IU\,ml^{-1}$ penicillin, 50 $\mu g\,ml^{-1}$ streptomycin, 0.3 $\mu g\,ml^{-1}$ amphotericin B, and 2 mM glutamine. The medium was replenished every 2 or 3 d. Cells were harvested after 1 wk using 0.25% trypsin, and inoculated at a concentration of 4×10^3 cells ml^{-1} into 24-well plates containing steam-sterilized set cement, or control discs of dense hydroxyapatite or polymethylmethacrylate (Acrylic, ICI UK), all discs were 12 mm diameter and 2 mm thick. Individual cultures were then maintained at 37 °C in an atmosphere of 5% CO_2 for a further week, the medium again being changed every 2 or 3 d. After 1 wk, cell growth was assessed by measuring the total protein content of individual cell cultures using Peterson's modification of the micro-Lowry method (Sigma Chemical Company, Poole, Dorset, UK). Briefly, an alkaline cupric tartrate complexed with the peptide bonds and formed a purple–blue color when the phenol reagent was added. Absorbance was read at 680 nm, and the protein concentration was determined from a calibration curve of known concentrations of bovine serum albumin. Cell viability was assessed by measuring the mitochondrial activity of the cells using an MTT assay. A solution of 3-[4,5-dimethylthiazol-2-*y*l]-2,5-diphenyl tetrazolium bromide or MTT (Sigma Chemical Company) was aseptically added to individual cell cultures in an amount equal to 10% of the culture medium volume. The cultures were incubated for 4 h, during which time the mitochondrial dehydrogenases of viable cells cleaved the tetrazolium ring of the MTT solution, yielding purple MTT formazan crystals. The crystals were then dissolved in isopropanol, and the resulting purple solution was measured spectrophotometrically at 570 nm. Mitochondrial activity was determined from a calibration curve produced using cultures of known varying numbers of viable cells. Statistical analysis was undertaken using the unpaired Student's *t*-test.

3. Results

3.1. Sodium and potassium ion release

For these alkali metal ICs, the amount of sodium released was directly related to the sodium ion content of the constituent glass; the release from LG5 (0.05 Na) and LG6 (0.1 Na) being significantly greater ($p < 0.05$) than the release from LG11 (0.05 Na/K) at 3 and 6 wk (see Table I). Similarly, the amount of potassium released was directly related to the potassium ion content of the constituent glass; the release from LG8 (0.0525 K) being significantly greater than the release from LG11 (0.05 Na/K) at 1, 3 and 6 wk (see Table II).

3.2. Fluoride ion release

Ionomer glasses containing increasing alkali metal cation concentrations, produced cements with increased total fluoride release profiles. LG2 (containing no monovalent cation) showed a low fluoride ion (F^-)

TABLE I Total accumulative sodium ion release (μmoles per g cement) from LG5, LG6 and LG11 (sodium-containing cements). Values are the mean ± standard deviation of three determinations

Ionomeric cement	Metal oxide content of glass	Total accumulate Na^+ release ($\mu mol\,g^{-1}$)		
		1 wk	3 wk	6 wk
LG5	0.05 Na	9.13 ± 0.47^a	19.11 ± 2.94^a	25.52 ± 2.37^a
LG6	0.1 Na	9.24 ± 0.98	23.11 ± 5.29^b	40.24 ± 6.29^b
LG11	0.05 Na/K	8.00 ± 0.70	14.01 ± 1.18	17.71 ± 1.02

$p < 0.05$, [a] LG5 compared to LG11, [b] LG6 compared to LG5 and LG11.

TABLE II Total accumulative potassium ion release from LG8 and LG11 (potassium containing cements). Values are the mean ± standard deviation of three determinations

Ionomeric cement	Metal oxide content of glass	Total accumulate K^+ release ($\mu mol\,g^{-1}$)		
		1 wk	3 wk	6 wk
LG8	0.0525 K	7.13 ± 0.40^a	13.92 ± 0.46^a	19.98 ± 0.66^a
LG11	0.05 Na/K	3.73 ± 0.38	7.25 ± 1.17	10.13 ± 1.26

[a] $p < 0.05$, LG8 compared to LG11.

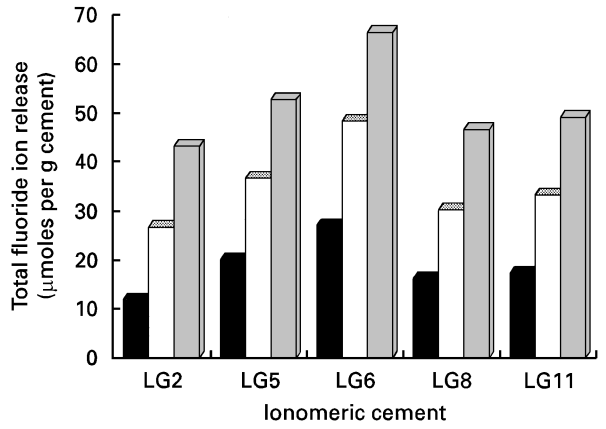

Figure 1 Accumulative total fluoride ion release. Values are the mean of three determinations; the standard deviations did not exceed 6 μmol g^{-1} cement. (■) 1 wk, (□) 3 wk, (▨) 6 wk.

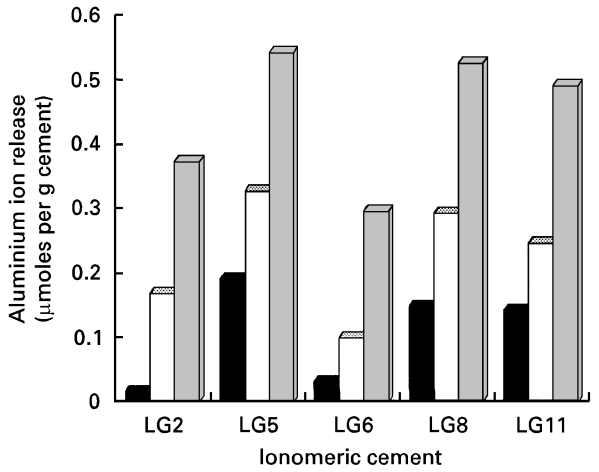

Figure 3 Accumulative aluminum ion release. Values are the mean of three determinations; the standard deviations did not exceed 0.02 μmol g^{-1} cement. (■) 1 wk, (□) 3 wk, (▨) 6 wk.

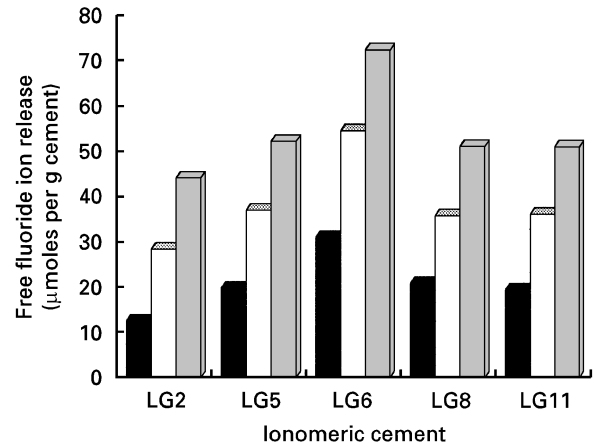

Figure 2 Accumulative free fluoride ion release. Values are the mean of three determinations; the standard deviations did not exceed 8 μmol g^{-1} cement. (■) 1 wk, (□) 3 wk, (▨) 6 wk.

TABLE III *In vitro* biocompatibility data. Values are the mean ± standard deviation of three or more determinations

Ionomeric cement	Total protein (μg ml^{-1})	MTT assay (10^5 cells)
LG2	111 ± 16[a,b]	11.3 ± 0.2[a]
LG5	73 ± 13	8.8 ± 1.5
LG6	96 ± 10[a]	11.5 ± 0.7[a]
LG8	87 ± 28	
LG11	88 ± 12	
Acrylic	94 ± 10	13.0 ± 0.5
Ha	127 ± 16	13.8 ± 1.5

$p < 0.05$, [a] LG2 and LG6 compared to LG5, [b] LG2 compared to LG6.

release rate, while LG6 (the material with the greatest monovalent cation concentration) released the most F^- ions (see Fig. 1). The order of fluoride release was LG6 > LG5 > LG11 > LG8 > LG2, where LG6 released significantly ($p < 0.05$) more fluoride than all the other glasses, and LG5 released significantly ($p < 0.05$) more fluoride than LG8 and LG2. The concentration of free fluoride was also determined using samples to which TISAB III was not added (see Fig. 2). These results were not significantly different ($p > 0.05$) to those for total fluoride, and it is possible that a little of the fluoride released in the early stages was bound to cations, e.g. ALF^{2+} species.

3.3. Aluminum ion release

The aluminum ion release did not correlate with fluoride ion release (see Fig. 3). The greatest release of aluminum was observed with LG5, and LG8, both of which contain neither the highest nor lowest monovalent cation concentrations. The lowest aluminum rates were recorded for LG6 and LG2. The order of aluminum release was LG5 > LG8 > LG11 > LG2 > LG6, where LG2 and LG6 released significantly ($p < 0.05$) less aluminum than the other three glasses, and where LG11 released significantly ($p < 0.05$) less aluminum than LG5.

3.4. *In vitro* biocompatibility

The results of protein determinations are shown in Table III. Three glasses were selected for testing by the MTT assay: LG6 (low aluminum ion release, high fluoride ion release), LG5 (highest aluminum ion release), and LG2 (containing no monovalent cation). The results of the MTT assay are also given in Table III. The cellular response to the glasses was similar but can be ordered as LG2 > LG6 > LG11 > LG8 > LG5 where LG2 was significantly ($p < 0.05$) better than LG6, and LG6 was significantly better than LG5 ($p < 0.05$). This gives an inverse relationship between the aluminum ion release from the ICs at 1 wk (LG5 > LG8 > LG11 > LG6 > LG2) and cellular growth/viability.

There was no correlation between the cellular response to the ICs studied and the fluoride ion release: LG6 > LG5 > LG11 > LG8 > LG2. Interestingly, LG6 had a low aluminum ion release in combination with a high fluoride ion release and showed a good cellular response, indicating that it may not be fluoride that is responsible for *in vitro* toxicity as shown by other studies [5]. The response of cells to Ha and

Acrylic confirmed the inert nature of these materials and their good *in vitro* biocompatibility.

4. Discussion

In the present study, we have shown that ion release from ICs is determined by the composition of the ionomer glasses used to make the ICs and the combinations of ions present. Increasing the alkali metal content of the constituent glass, not only increased the rate and amount of monovalent cation released from the cement matrix, but also the rate and amount of fluoride released. This was probably due to the alkali metal ions facilitating an ion exchange mechanism between the cement and the environment, by increasing the mobility of fluoride ions within the cement matrix. This is important, because we have shown previously that the mobility of ions within the cement matrix and their release characteristics, are significant factors affecting the biological response to ICs *in vivo* [24].

Early work into the bioactivity of ICs and their effects on osteogenic cells demonstrated that the composition of ICs was important, with non-fluoride glasses being the least toxic to cells *in vitro* [5] but the least osteoconductive to cells *in vivo* [11]. The beneficial effects of fluoride are thought to be due to promotion of osteoblastic activity and increasing trabecular bone density [25], and it is used in the treatment of osteoporosis to inhibit bone resorbtion [17, 26]. The effects of fluoride, however, appear to be dose-dependent *in vitro* [15, 27] and *in vivo* [17, 28]. *In vitro*, at relatively high concentration, fluoride acts as an enzyme inhibitor, but *in vivo* it stimulates proliferation and alkaline phosphatase activity of bone-forming cells [11, 15, 16]. The role of other ions, particularly aluminum is more controversial [29]. In the present study, we have found that aluminum ion release from ICs has an inverse relationship to *in vitro* biocompatibility. It is possible that it is this ion that is the dominant ion determining *in vitro* biocompatibility rather than fluoride, as was originally proposed [5]. Aluminum may, however, like fluoride act in a dose-dependent manner. Low concentrations of aluminum ions have been shown to stimulate the proliferation of osteoblasts and new bone formation *in vitro* and *in vivo* [14, 18]. At high concentrations, aluminum has been shown to inhibit bone mineralization [20]. It has also been reported to enhance the mobilization of calcium from bone by a cell-independent mechanism, interfere with the initial stages of crystallization of calcium phosphate *in vivo*, and exert an indirect effect on bone formation through the inhibition of collagen synthesis [20, 22, 30, 31]. The mechanism(s) underlying these disparate effects of aluminum are unknown. The complexity of the aluminum osseous actions may result from differential responses of pre-osteoblasts and mature osteoblasts to aluminum and/or modulation of these direct cellular effects by interactions with systemic and/or local factors such as parathyroid hormone, vitamin D and/or other bone growth factors [21]. In addition, differences in speciation of aluminum could contribute to its differential actions.

5. Conclusion

Increased aluminum levels in bone undoubtedly exert a variety of established clinical toxic effects, such as vitamin-D resistant osteomalacia and diminished bone remodeling activity [20–22]. When, however, aluminum administration in animals results in defective bone mineralization, cure of osteomalacia can often be achieved by subsequent administration of vitamin D [21]. Thus, the precise effects of aluminum on bone and the underlying mechanisms involved remain an area for debate [21].

In this study, it appeared that aluminum ions contributed to the *in vitro* toxicity of ICs. This was, however, a dose-dependent effect, and those cements which released less aluminum were more biocompatible in the bone cell model used. The implication for biomedical ICs, particularly those intended for bone tissue repair and orthopaedics, is that these materials should be based on glasses which produce cements with reduced aluminum ion release profiles. These observations are in accordance with recent *in vivo* studies, particularly those which linked mineralization defects in bone around IC implants to aluminum ion release [32]. Further work might, therefore, be usefully directed at the design of ICs with significantly reduced or no aluminum ion release.

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