In vitro Cytotoxicity of Amorphous Calcium Phosphate Composites*†

C. G. Simon Jr,[1] J. M. Antonucci,[1] D. W. Liu[1] and D. Skrtic[2,#]
[1]*Polymers Division, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA*
[2]*Paffenbarger Research Center, American Dental Association Foundation, Gaithersburg, MD 20899, USA*

ABSTRACT: Calcium phosphate-based biomaterials are being increasingly used as bone substitutes in dentistry and in reconstructive and orthopedic applications because of their good biocompatibility, osteoconductivity and/or bone-bonding properties. In this study, the *in vitro* cytotoxicity of the amorphous calcium phosphate (ACP) filler, the copolymer matrix derived from the polymerization of a resin system and the corresponding ACP composite was analyzed utilizing cell culture techniques. The photo cured polymer was derived from an activated resin comprised of an ethoxylated bisphenol A dimethacrylate, urethane dimethacrylate, triethylene glycol dimethacrylate, and 2-hydroxyethyl methacrylate. The resin was admixed with a zirconia-ACP filler to prepare the composite. Specimens were extracted in media overnight and then MC3T3-E1 osteoblast-like cells were cultured in the extracts for 3 days. Cytoxicity was evaluated by phase contrast microscopy and an enzymatic assay for mitochondrial dehydrogenase activity (Wst-1). Cellular response to the experimental ACP composite was compared to the cellular response of commercially available light-cure orthodontic adhesive. In addition to the cytotoxicity testing the ion release profiles of ACP composites was determined. Furthermore, a degree of vinyl conversion (DVC) attained in the experimental composite and in the commercial control was compared. No adverse response regarding cell morphology and/or viability was observed with ACP composites

#Author to whom correspondence should be addressed:
E-mail: drago.skrtic@nist.gov
*No benefit of any kind will be received either directly or indirectly by the authors.

0883-9115/04/06 0439–14 $10.00/0 DOI: 10.1177/0883911505051854

compared to the unfilled copolymers or to the commercial adhesives. Sustained release of potentially remineralizing calcium and phosphate ions and favorable DVC of these composites confirms their value in a variety of dental and possibly orthopedic applications where anti-demineralizing/remineralizing efficacy is the primary goal.

KEY WORDS: amorphous calcium phosphate, composites, cellular response, cytotoxicity, degree of vinyl conversion, extract test, ion release profiles.

INTRODUCTION

The main advantages of calcium phosphate (CP)-based biomaterials designed for dental and/or orthopedic bone tissue regeneration are their osteoconductivity and biocompatibility [1–3]. The latter arises from their chemical composition which resembles that of the inorganic phase of natural bone tissues. CPs with solubility above that of hydroxyapatite (HAP) are reactive and expected to contribute to bone formation by osteoblasts [4]. We have recently focused on one of the more soluble CPs, ACP. When embedded in polymerized methacrylate matrices [5,6] and exposed to an aqueous environment, ACP releases sufficient levels of calcium and phosphate ions in a sustained manner to promote redeposition of thermodynamically stable, apatitic tooth mineral [7]. A problem with dental composites of all types is their inability to resist cracking under masticatory stress due to their low strength and toughness. This is particularly so in the case of ACP composites which are mechanically inferior to glass-filled composites. To overcome this shortcoming we have focused on several strategies for improving the ACP filler/polymer matrix interfacial properties (as well as composite properties) by controlling the particle size distribution and surface properties of ACP fillers and/or by fine-tuning of the resin [8–10].

It has been documented in the literature that biocompatibility and osteoconductivity of CP biomaterials vary with the type of CP utilized [2,11–15]. However, despite considerable research efforts, the mechanism by which the more soluble CPs promote osteogenesis remains unclear [4,16–18]. We have found that copolymers derived from highly converted resins, e.g., ethoxylate bisphenol A dimethacrylate (EBPADMA) as a base monomer and triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxylethyl methacrylate (HEMA) as comonomers, also yield polymeric ACP composites with high degrees of vinyl conversion (DVC) (i.e., the low leachability of unreacted monomeric species which is taken as an indirect measure of high biocompatibility) and favorable ion (calcium and phosphate) release profiles [10]. In this study, the cytotoxicity of bioactive ACP composites

has been assessed in an attempt to shed light on the possible interactions between this relatively soluble filler and osteoblast-like cells. To test the hypothesis that ACP has no adverse effect on cell morphology and/or viability, an osteoblastic cell culture system MC3T3-E1 [19] was exposed to extracts of the ACP filler, the experimental copolymers and their ACP composites [20]. Additionally, cellular response to extracts of our experimental composites was compared to commercially available orthodontic adhesive of similar matrix composition. Cytotoxicity data were correlated with DVC and the kinetics of the release of the remineralizing calcium and phosphate ions from the composites.

MATERIALS AND METHODS

Synthesis, Milling and Evaluation of ACP Filler

Zirconia-hybridized ACP (Zr-ACP) filler was synthesized by utilizing a modified preparation protocol of Eanes et al. [21]. Zr-ACP precipitated instantaneously in a closed system at 23°C upon rapidly mixing equal volumes of a 800 mmol/L $Ca(NO_3)_2$ solution, a 536 mmol/L Na_2HPO_4 solution that contained a molar fraction of 2% $Na_4P_2O_7$ as a stabilizing component for ACP, and an appropriate volume of a 250 mmol/L $ZrOCl_2$ solution (mole fraction of 10% $ZrOCl_2$ based on Ca reactant). The reaction pH varied between 8.6 and 9.0. The suspension was then filtered, the solid phase was washed with ice-cold ammoniated water and acetone and the product was lyophilized.

Wet milling of the lyophilized Zr-ACP was performed as follows: the Zr-ACP powder was mixed with very high density ZrO_2 balls (2 mm in diameter; Glen Mills Inc., Clifton, NJ, USA) at ZrO_2/Zr-ACP mass ratio of approximately 20: with isopropanol added in an amount sufficient to cover the mixture. The milling (ball-milling machine, Dayton Electric MFG Co., Chicago, IL, USA) was performed at 57 rad/s (540 rpm) for 2.5 h. The Zr-ACP solid slurry was then separated from the ZrO_2 balls by sieving, the isopropanol was removed by rotary evaporation (approximately 2 h at 50°C) and finally the milled filler was dried in a vacuum-oven at 40°C for 24 h. Milled Zr-ACP was kept in a desiccator under a moderate vacuum (2.7 kPa) until utilized in the preparation of composites.

The amorphous state of milled ACP was verified by powder X-ray diffraction (XRD: Rigaku X-ray diffractometer, Rigaku/USA Inc., Danvers, MA, USA; standard uncertainty of the measured d-values within 0.05% of the reported values of NIST SRM® 640 silicon powder) and Fourier-transform spectroscopy (FTIR: Nicolet Magna-IR FTIR System 550 spectrophotometer, Nicolet Instrument Corporation,

Madison, WI, USA). Morphology/topology of Zr-ACP particles was examined by scanning electron microscopy (SEM; JSM-5400 instrument, JEOL Inc., Peabody, MA, USA). The particle size distribution (PSD) was determined by gravitational/centrifugal sedimentation analysis (SA-CP3 particle size analyzer, Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) following dispersion of the solids in isopropanol by ultrasonication for 10 min. Water content of the fillers was determined by thermogravimetric analysis (TGA; Perkin Elmer 7 Series Thermal Analysis System, Norwalk, CT, USA). The TGA was performed by heating 5 to 10 mg of the filler at the rate of 20°C/min over a temperature range of 30 to 600°C in air. Ca/PO_4 ratio of the solids after dissolution in HCl was calculated from solution Ca^{2+} and PO_4^{-3} values [UV/VIS Carey Model 219 spectrophotometer (Varian Analytical Instruments, Palo Alto, CA, USA)] [22,23]. Results of the physicochemical characterization of the milled Zr-ACP filler are summarized in Table 1 and Figure 1(a)–(c).

Formulation of the Resin

The experimental resin was formulated from commercially available dental monomers and activated with a visible light initiator system consisting of camphorquinone and ethyl-4-N,N-dimethylaminobenzoate (Table 2). The acronyms indicated in Table 2 will be used throughout this manuscript.

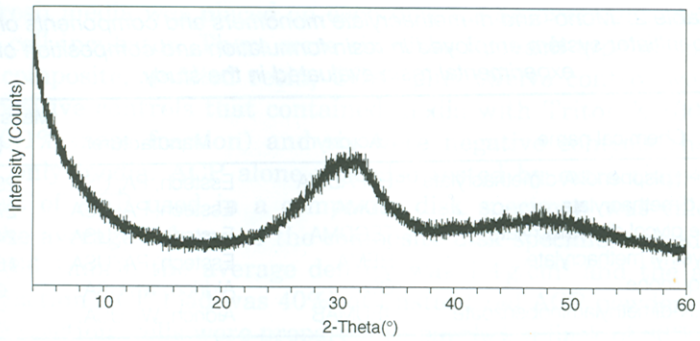
Preparation of Composite and Copolymer Disk Specimens

The first step in ACP composite preparation involved blending the liquid resin (mass fraction 60%) and milled Zr-ACP filler (mass fraction 40%) by hand spatulation. Once a homogeneous consistency of

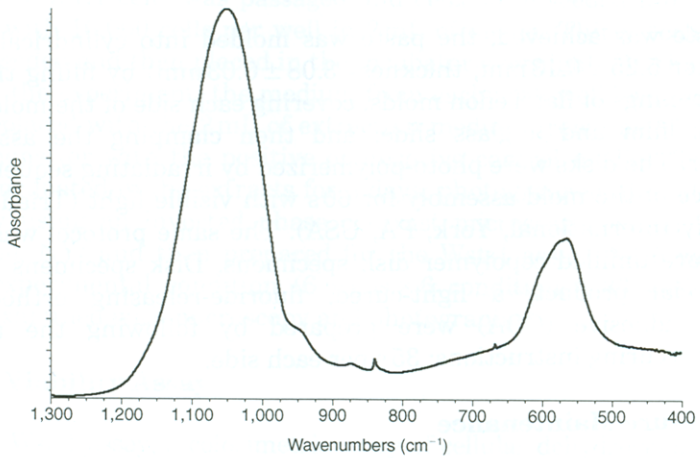
Table 1. Physicochemical characteristics of milled Zr-ACP filler utilized in the study.

Parameter	Value*
Particle size range (μm)	0.2 to 3.0
Median particle diameter, d_m (μm)	0.9 ± 0.2
Specific surface area, SSA (m^2/g)	3.8 ± 1.0
Water content (mass fraction, %)	16.9 ± 1.4
Calcium/phosphate ratio**	1.89 ± 0.10

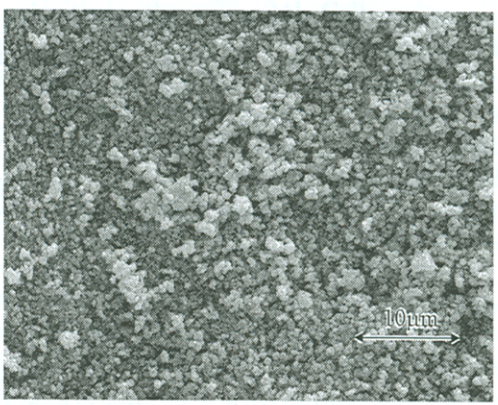
*Except for the particle size range, results represent mean value ± SD. The number of repetitive runs in each group $6 \geq n \geq 3$.
**Due to the co-precipitation of HCl-insoluble amorphous Zr-phosphate (mass fraction 21.9%). Theoretical (expected) value: 1.50.



(a)



(b)



(c)

Figure 1. XRD pattern (a), FTIR spectrum (b) and the SEM image (c) of milled Zr-ACP filler utilized in the study.

Table 2. Mono- and di-methacrylate monomers and components of photo-initiator system employed in resin formulation and composition of the experimental resin evaluated in the study.

Chemical name	Acronym	Manufacturer	Mass fraction (%)
ethoxylated bisphenol A dimethacrylate	EBPADMA	Esstech, PA, USA	28.07
urethane dimethacrylate	UDMA	Esstech, PA, USA	27.99
triethylene glycol dimethacrylate	TEGDMA	Esstech, PA, USA	24.97
2-hydroxyethyl methacrylate	HEMA	Esstech, PA, USA	16.81
camphorquinone	CQ	Aldrich, WI, USA	0.40
ethyl-4-N,N-dimethylaminobenzoate	4EDMAB	Aldrich, WI, USA	0.79

the paste was achieved, the paste was molded into cylindrical disks (diameter 5.25 ± 0.13 mm; thickness 3.08 ± 0.03 mm) by filling the circular openings of flat Teflon molds, covering each side of the mold with a Mylar film and a glass slide, and then clamping the assembly together. The disks were photo-polymerized by irradiating sequentially each face of the mold assembly for 60 s with visible light (Triad 2000, Dentsply International, York, PA, USA). The same protocol was used to prepare unfilled copolymer disk specimens. Disk specimens of the commercial product, a light-cured, fluoride-releasing orthodontic bracket adhesive (COA) were prepared by following the manufacturer's curing instructions: 35 s per each side.

Cell Culture Maintenance

MC3T3-E1 cells (Riken Cell Bank, Hirosaka, Japan) were maintained in α-modification of Eagle's minimum essential medium (Biowhittaker, Walkersville, MD, USA) with a volume fraction of 10% fetal bovine serum (Gibco-BRL-Life Technologies, Rockville, MD, USA) and 60 mg/L kanamycin sulfate (Sigma, St Louis, MO, USA) in a fully humidified atmosphere with a volume fraction of 5% CO_2 at 37°C. The medium was changed twice a week. Cultures were passaged with EDTA-containing (1 mmol/L) trypsin solution (mass fraction of 0.25%; Gibco, Rockville, MD, USA) once a week.

Extract Experiments

During the first 24 h of the experiment, all disk specimens were sterilized in a 50 mL tube containing 25 mL of 70% ethanol for 5 min and then placed in 24-well plates (BD Biosciences, Bedford, MA, USA) with one disk per well. Each disk was washed with 2 mL of media for 1 h and

then fresh media was placed on each disk for an overnight extraction in the cell incubator. There were six disks for each type of specimen (ACP composite, unfilled resin, COA) plus twelve control wells. Six were positive controls that contained media with Triton X-100 detergent (0.1% mass fraction) and six were negative controls that contained only media. ACP alone was also tested by extracting 37.5 mg (amount of ACP used in a composite disk specimen was calculated from the average volume of the composite disk specimens used in the study (66.6 mm^3), the average density was 1.4 g/cm^3 and the average mass fraction ACP load was 40%) of unsterilized ACP powder. A total of 30 extraction wells were prepared. In parallel, a flask of 80% confluent MC3T3-E1 cells was passaged and cells were seeded into 24-well plates with 10,000 cells per well in 2 mL of media. Thirty "cell wells" were seeded and then placed in the incubator overnight. On the second 24 h of the experiment, the medium from each "cell well" was removed and replaced with the 2 mL of extraction medium from one of the disk specimens (or with the positive or negative control media). The cells were incubated in the extracts for 3 days, photographed (digital photography using an inverted phase contrast microscope, Nikon TE300, Melville, NY) and then prepared for the Wst-1 assay. All 6 wells for each experimental condition (6 wells $\times$ 6 conditions = 36 wells total) were examined with microscopy and photographed.

Wst-1 Viability Assay

The Wst-1 assay, a colorimetric assay of cellular dehydrogenase activity, was used (the absorbance at 450 nm is proportional to the amount of dehydrogenase activity in the cell) [24]. Cells cultured in the extracts were rinsed with 1 mL of Tyrode's HEPES buffer (140 mmol/L NaCl, 0.34 mmol/L Na_2HPO_4, 2.9 mmol/L KCl, 10 mmol/L HEPES, 12 mmol/L $NaHCO_3$, 5 mmol/L glucose, pH 7.4). One mL of Tyrode's HEPES buffer and 0.1 mL of Wst-1 solution (5 mmol/L Wst-1 and 0.2 mmol/L 1-methoxy PMS in water) were then added to each well. After a 2 h incubation at 37°C, a 0.2 mL aliquot from each well was transferred to a 96-well plate and absorbance was measured with a plate reader (Wallac 1420 Victor^2, PerkinElmer Life Sciences, Gaithersburg, MD). Blank wells that contained only Tyrode's HEPES buffer and Wst-1 solution, were also prepared, incubated and read. The blank value was subtracted from each of the experimental values as background. Wst-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) and 1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate) were obtained from Dojindo (Gaithersburg, MD).

Degree of Vinyl Conversion (DVC)

Fourier-transform infrared (FTIR) spectroscopy was used to determine the DVC of the experimental ACP composite and the COA. DVC was calculated from the decrease in the integrated peak area for the vinyl group (1,637 cm^{-1} absorption band) against that of the phenyl ring (1,582 cm^{-1} absorption band) in going from the unpolymerized (monomer) to polymerized composites (polymer) using the following expression:

$$DVC = \{1 - [area(1637/1582)_{polymer}/area(1637/1582)_{monomer}]\} \times 100 \quad (1)$$

FTIR spectra were acquired by collecting sixty-four scans at wavelength accuracy $\leq 0.01\,cm^{-1}$ at 2,000 cm^{-1}. A minimum of five repetitive measurements were performed for each experimental group.

Kinetics of Ion Release from ACP Composites

Mineral ion release from each individual ACP composite disk specimen (six replicate runs) was examined in continuously stirred, HEPES-buffered (pH = 7.40) saline solutions (ionic strength = 0.13 mol/L) at 37°C. Ca^{2+} and PO_4 levels released into solution were determined colorimetrically [22,23] at predetermined time intervals (up to 790 h). Ion-release data were corrected for variations in the total surface area of the disk exposed to saline solution. Normalized value for a given surface area, A (in mm^2) was calculated as follows; normalized value = (measured value) × (500/A).

The thermodynamic stability of the saline solutions containing the maximum concentrations of ions released from the disk was calculated with respect to stoichiometric apatite (HAP; $Ca_{10}(OH)_2PO_4)_6$) by using the Gibbs free-energy expression [10]:

$$\Delta G^\circ = -2.303(RT/n)\ln(IAP/K_{sp}) \quad (2)$$

where IAP is the solution ion activity product for HAP (calculated by using the Chemist software, MicroMath Research, St Louis, MO, USA), K_{sp} is the thermodynamic solubility product of the HAP, R is the ideal gas constant, T is the absolute temperature, and $n = 18$ is the number of ions in the IAP. Negative ΔG° value indicates the solution supersaturated with respect to HAP.

Statistical Analysis of the Experimental Data

Experimental data were analyzed by ANOVA ($\alpha = 0.05$). Significant differences between specific groups were determined by Turkey's pair-wise comparisons (95% confidence interval). One standard deviation is indicated as a measure of the standard uncertainty of the measurements.

RESULTS

Cell Morphology

Negative control cells cultured in only media displayed a normal, spread, polygonal morphology (Figure 2(e). Cells cultured in ACP or

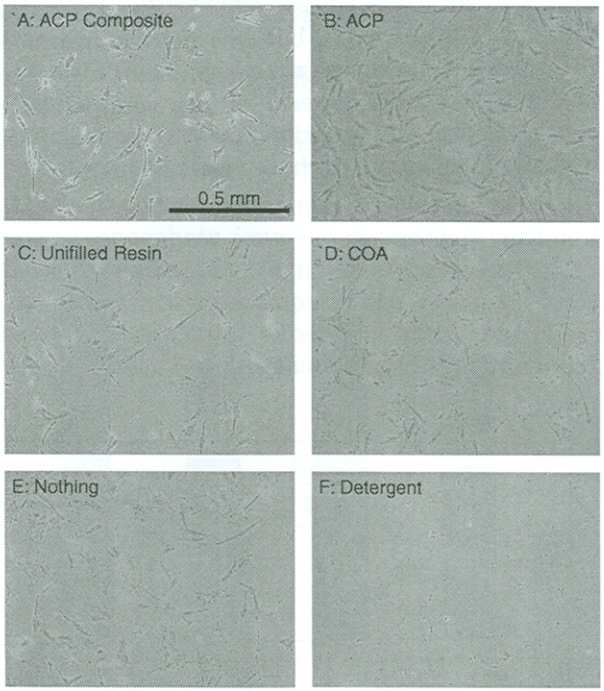


Figure 2. Representative phase contrast images of MC3T3-E1 cells cultured in extracts from ACP powder and different resin composites for 3 days. The size bar in the bottom of panel A applies to all panels. "Unfilled resin" is EBPADMA/UDMA/TEGDMA/HEMA copolymer, "COA" is a light-cure, fluoride releasing orthodontic adhesive, "Nothing" is a negative control and "Detergent" is a positive control (6 wells were examined and photographed for each experimental condition).

resin extracts (Figures 2(a)–(d)) had a polygonal morphology similar to negative controls (Figure 2(e)) irrespective of the extract in which they were cultured (Figures 2(a)–(d)). The presence of only cell remnants in Figure 2(f) indicates that 0.1% detergent is cytotoxic which serves as a positive control for the assay. Qualitative comparison of the negative control panel (Figure 2(e)) with the experimental panels (Figures 2(a)–(d)) reveals that there are approximately an equivalent amount of cells in each of the panels which suggests no adverse cellular response to ACP powder, copolymer (unfilled resin), ACP composite and COA.

Cell Viability

Based on the Wst-1 assay (Figure 3) of the extracts from the resins caused a mild drop in the viability of MC3T3-E1 cells compared to the negative control. This drop was statistically significant for ACP powder, ACP composite and COA ($p < 0.05$) but was not significant for unfilled resin ($p > 0.05$). However, there was not a significant difference between ACP composite and the unfilled resin or the commercial composite. These results suggest that the experimental ACP composite is no more toxic than the commercial adhesive chosen as the control.

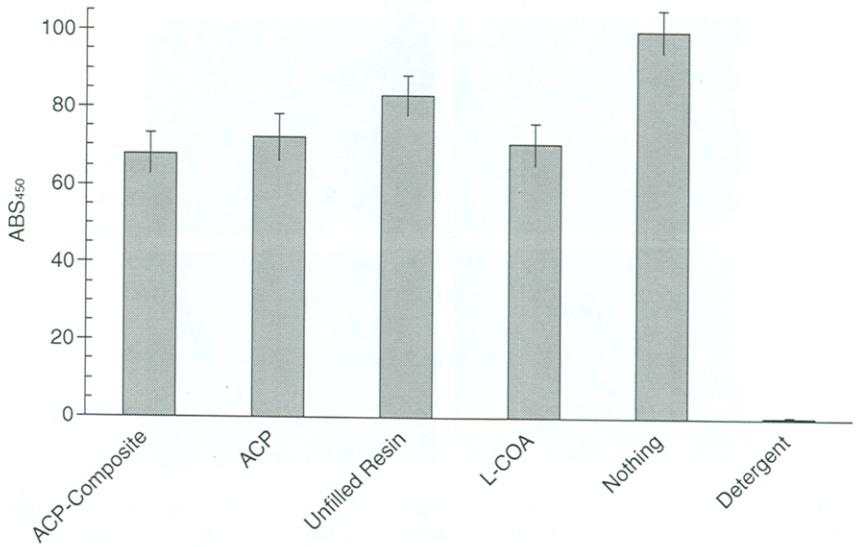


Figure 3. Cell viability (Wst-1 assay) for the experimental ACP composite, ACP powder, unfilled resin (copolymer) and COA compared to negative (Nothing) and positive control (Detergent) (number of samples in each group was $n = 6$).

Degree of Vinyl Conversion

The mid-FTIR conversion results for the experimental ACP composite and the COA are presented in Figure 4. The attained DVC of the ACP composite was consistently higher than the DVC of COA at 1 h and 24 h post-cure (29% and 24%, respectively).

Ion Release Profiles

Milled ACP-filled EBPADMA/UDMA/TEGDMA/HEMA composites steadily released mineral ions upon immersion in buffered saline; the kinetic profiles are given in Figure 5. The Ca^{2+} and PO_4^{-3} released were sufficient to maintain a favorable remineralization potential, i.e. the required supersaturation for the internal (intracomposite) reprecipitation of apatite to occur (the average $\Delta G° = -4.92$ kJ/mol).

DISCUSSION

CP biomaterials are biocompatible because the main inorganic constituent of bone, hydroxyapatite (HA), is comprised of calcium and phosphate. HA is the main inorganic constituent of hard tissues and free calcium and phosphate ions can be used in metabolism [3,25]. Reported biocompatibility of various CP cements [2,12,13,15,26–30] is attributed to either the biocompatibility of their individual constituents [α- and β-tricalcium phosphate, dicalcium phosphate dihydrate (DCPD), tetracalcium phosphate and HA] or the biocompatibility

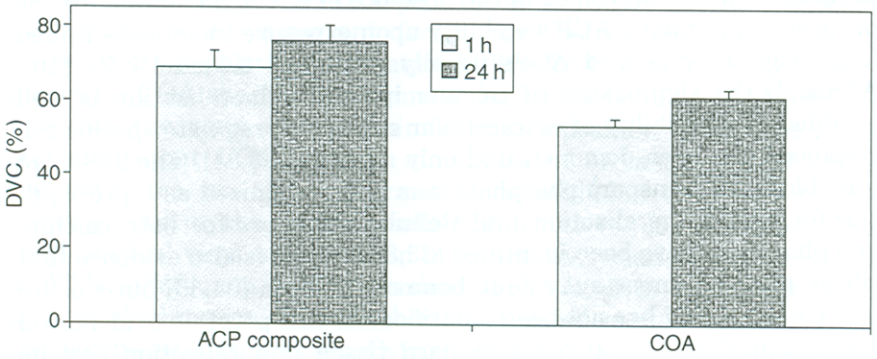


Figure 4. Mean values and standard deviation (indicated by bars) of the DVC of the experimental ACP composite and COA attained 1 h and 24 h after curing (number of runs in each experimental group was $n \geq 5$).

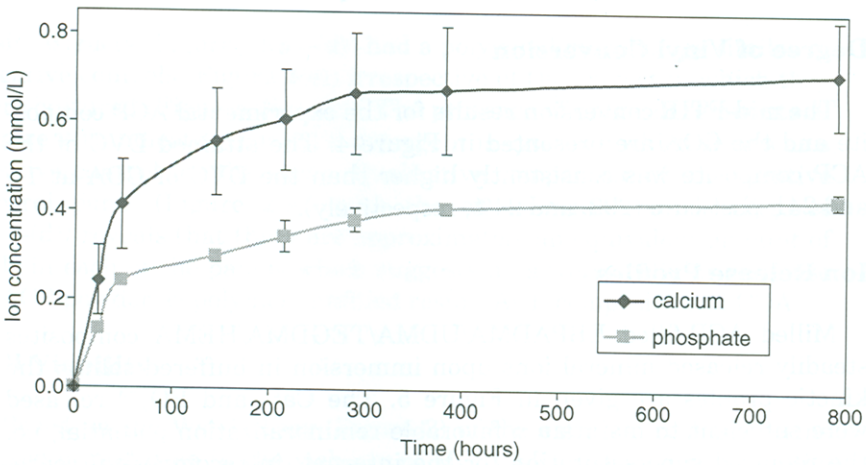


Figure 5. Kinetic profiles of Ca^{2+} and PO_4 ion release (mean value ± standard deviation) from the experimental ACP composites (number of repetitive runs was $n = 6$).

of the reaction product(s) (predominantly calcium-deficient HA, traces of DCPD). It is, therefore, of no surprise that ACP (best described by the compositional formula $Ca_3(HPO_4)_{0.195}(PO_4)_{1.87}$ for pH 7.4 to 9.0 [31]) itself as well as the ACP-based composite had only a mild toxic effect on MC3T3-E1 cells.

It is important to mention that in preparing the fast-setting CP cements described in the literature, no ions other than calcium, phosphate, sodium and/or potassium were utilized. As detailed in the Materials and Methods section, the ACP used in our experimental composites was synthesized in the presence of zirconyl chloride for the purpose of improving ACP's stability upon exposure to aqueous milieu [32]. Such Zr-stabilized ACPs usually contained 8.6 ± 1.4% Zr [10]. Although the significance of calcium in bone mineralization is well established, the ability of extracellular calcium to regulate specific cell responses has been demonstrated only recently [33,34]. The ability of osteoblasts to transport phosphate was also recognized as a prerequisite for bone mineralization [36] Cellular receptors for both calcium and phosphate have been identified [33,35]. There is also evidence that silicon plays an important role in bone metabolism [34,36] but a cellular receptor for Si has not been identified. At this point no evidence on the potential role of zirconia in hard tissue mineralization and its interaction(s) is available. It is, however, possible that the coprecipitation of Zr with the ACP could have some effect on mitochondrial dehy-

drogenase activity of cells cultured in the extract of Zr-ACP-filled EBPADMA/UDMA/TEGDMA/HEMA matrices. A series of cell viability experiments utilizing non-stabilized ACP (ACP synthesized without any hybridizing/stabilizing ion) would be necessary to test this possibility.

Polymerization of dental resin composites is usually less complete than that of the unfilled resin, and almost every component can be detected in the extracts of polymerized materials [37,38]. Some of the released, unpolymerized resin monomers may elicit various biological effects such as genetic mutations *in vitro* [39]. Among commonly used methacrylate monomers, TEGDMA has been reported as directly mutagenic in a mammalian cell gene mutation assay while no mutagenic effects were detected with UDMA and HEMA. No information was available for EBPADMA, a base monomer utilized in our experimental copolymer and composite. On the other hand, cytotoxicity of the resin components of composites and adhesives (expressed as a concentration that suppresses the mitochondrial activity by 50%, i.e., TC50 concentration) was ranked as follows UDMA > TEGDMA > HEMA after 72 h exposure to Balb/c 3T3 mouse fibroblasts [40]. In summary, it is likely that the mild effects of the resins on cell viability observed in this study are a result of the effects of leachable monomers or initiator components or byproducts on cells.

There are a number of factors that determine the cytotoxicity of dental polymers and composites. The chemical-structure property relations of the monomers in the resin system are undoubtedly involved in determining cellular response. Furthermore, compositional differences involving polymers and photoinitiator systems can be a significant contributor. Finally, the DVC, especially as it relates to the leachable monomers, is an important factor that determines the cytotoxic response of the polymerized materials. The DVC attained in ACP composite vs. COA specimens (Figure 4) may be attributed to the differences in the resin and photoinitiator composition (other than the statement that COA resin is a mixture of aromatic and aliphatic methacrylates no other information is available from the manufacturer) and/or the curing conditions (1 min vs. 35 s per side). The total residual vinyl unsaturation from photopolymerization, measured by FTIR (DVC), consisted of pendent vinyl groups in the matrix phase plus residual monomers and other unsaturated species that arise from the polymerization process. Cytotoxicity is more likely to depend on leachable residual monomers and other leachable organic species in the composite. Therefore, for ACP composites, and traditional dental composites, the correlation of cytotoxicity with the extent of vinyl

conversion requires the assessment of leachable organic moieties as well as the total vinyl groups in the composite. Therefore, the results presented in this study can only be taken as an assessment of the *in vitro* cytotoxicity of these materials. In future studies we will assess how the monomer system and the photopolymerization process affect the concentration of leachable moieties and presumably the cytotoxicity of these composites.

CONCLUSION

The results of this study show no adverse effects on cellular morphology or viability with ACP composites compared to the commercial orthodontic adhesive chosen as a control. The observed mild reduction in viability may be attributed to the leachability of unreacted resin components rather than the cytotoxicity of ACP filler itself. Favorable degrees of vinyl conversion and sustainable release of mineral ions coupled with the satisfactory cellular response make these bioactive composites a promising tool in regenerative hard tissue applications where redeposition of the mineral is of primary concern.

DISCLAIMER

Certain commercial materials, instruments and/or equipment are identified in this work for adequate definition of the experimental procedures. In no instance does such identification imply recommendation or endorsement by the American Dental Association Foundation or the National Institute of Standards and Technology, nor does it imply that the materials, instruments and/or the equipment identified is necessarily the best available for the purpose.

ACKNOWLEDGEMENTS

Support for this research was provided from the grant 2R01 DE 013169-05 from the National Institute of Dental and Craniofacial Research to Dr Skrtic and in part by NIST. We gratefully acknowledge gifts of the EBPADMA, UDMA, TEGDMA and HEMA monomers from Esstech, Essington, PA, USA.

REFERENCES

1. Hollinger, J.O., Brekke, J., Gruskin, E. and Lee D. (1996). Role of Bone Substitute. *Clin. Orthop.*, **324**: 55–66.

2. LeGeros, R.Z. and LeGeros, J.P. (1996). Calcium Phosphate Biomaterials in Medical Application, *Bioceramics*, **9**: 7–10.
3. Yuan, H., Li, Y., de Bruijn, J.D., de Groot, K. and Zhang, X. (2000). Tissue Responses of Calcium Phosphate Cement: A Study in Dogs, *Biomaterials*, **21**: 1283–1290.
4. Ehara, A., Ogata, K., Imazato, S., Ebisu, S., Nakano, T. and Umakoshi, Y. (2003). Effects of α-TCP and TetCP on MC3T3-E1 Proliferation, Differentiation and Mineralization, *Biomaterials*, **24**: 831–836.
5. Antonucci, J.M., Skrtic, D., Hailer, A.W. and Eanes, E.D. (2001). Bioactive Polymeric Composites Based on Hybrid Amorphous Calcium Phosphate, In: Ottenbrite, R.M. and Kim, S.W. (eds), *Polymeric Drugs and Drug Delivery Systems, Technomics* Publ. Co., Inc., Lancaster, PA, pp. 301–310.
6. Skrtic, D., Antonucci, J.M., Eanes, E.D., Eichmiller, F.C. and Schumacher, G.E. (2000). Physicochemical Evaluation of Bioactive Polymeric Composites Based on Hybrid Amorphous Calcium Phosphates, *J. Biomed. Mat. Res. (Appl. Biomater.)*, **53**: 381–391.
7. Skrtic, D., Eanes E.D. and Antonucci, J.M. (1995). Polymeric Calcium Phosphate Composites with Remineralization Potential in *Industrial Biotechnological Polymers*, Gebelein, C.G. and Carraher, C.E. Jr. (eds), 393–408, Technomics Publ. Co., Lancaster, PA.
8. Skrtic, D., Hailer, A.W., Takagi, S., Antonucci, J.M. and Eanes E.D. (1996). Quantitative Assessment of the Efficacy of Amorphous Calcium Phosphate/Methacrylate Composites in Remineralizing Caries-like Lesions Artificially Produced in Bovine Enamel, *J. Dent. Res*, **75**(9): 1679–1686.
9. Skrtic, D., Antonucci, J.M., Eanes, E.D. and Eidelman, N. (2004). Dental Composites Based on Hybrid and Surface-modified Amorphous Calcium Phosphates. *Biomaterials*, **25**: 1141–1150.
10. Skrtic, D., Antonucci, J.M. and Eanes, E.D. (2003). Amorphous Calcium Phosphate-Based Bioactive Polymeric Composites for Mineralized Tissue Regeneration, *J. Res. Natl Inst. Stands. Technol*, **108**: 167–182.
11. St. John, K.R., Zardiackas, L.D., Terry, R.C., Teadsall, R.D. and Cooke, SE. (1995). Histological and Electron Microscopic Analysis of Tissue Response to Synthetic Composite Bone Graft in the Canine, *J. Appl. Biomater.*, **6**: 89–97.
12. Oonishi, H., Kushitani, S., Iwaki, H., Saka, K., Ono, H., Tamura, A., Sugihara, T., Hench, L.L., Wilson, J. and Tsuji, E. (1995). Comparative Bone Formation in Several Kinds of Bioceramic Granules, *Bioceramics,* **8**: 137–144.
13. Drissen, F.C.M., Boltong, M.G., Zapatero, M.I., Verbeeck, R.M.H., Bonfield, W., Bermudez, O., Fernandez, E., Ginebra, M.P. and Planell, J.A. (1995). *In vivo* Behavior of Three Calcium Phosphate Cements and a Magnesium Phosphate Cement, *J. Mater. Sci.: Mater. Med.*, **6**: 272–278.
14. Nakamura, T. (1996). Bioceramics in Orthopedic Surgery, *Bioceramics*, **9**: 31–34.

15. TenHuisen, K.S. and Brown, P.W. (1997). Variation in Solution Chemistry During Calcium-deficient and Stoichiometric Hydroxyapatite Formation from $CaHPO_4 2H_2O$ and $Ca_4(PO_4)_2O$, *J. Biomed. Mater. Res.*, **36**: 233–241.
16. Constantino, P.D., Friedman, C.D., Jones, K., Chow, L.C. and Sisson, G.A. (1992). Experimental Hydroxyapatite Cranioplasty, *Plast. Reconstr. Surg.*, **90**: 174–191.
17. Kurashina, K., Kurita, H., Kotani, A., Takeuchi, H. and Hirano, M. (1997). *In Vivo* Study of a Calcium Phosphate Cement Consisting of α-tricalcium Phosphate/dicalcium Phosphate Dibasic/tetracalcium Phosphate Monoxide, *Biomaterials*, **18**: 147–151.
18. Wiltfang, J., Merten, H.A., Schlegel, K.A., Schiltze-Mosgau, S., Kloss, F.R., Rupprecht, S. and Kessler, P. (2002). Degradation Characteristics of α and β Tri-calcium Phosphate in Minipigs, *J. Biomed. Mater. Res.*, **63**: 115–121.
19. Sudo, H., Kodama, H.-A., Magai, Y., Yamamoto, S. and Kasai, S. (1983). *In Vitro* Differentiation and Calcification in a New Clonal Osteogenic Cell Line Derived from Newborn Mouse Calvaria, *J. Cell. Biol.*, **96**: 191–198.
20. International Standards Organization. ISO 10993–5. Biological Evaluation of Medical Devices – Part 5. Tests for *in vitro* Cytotoxycity.
21. Eanes, E.D., Gillessen, H. and Posner, A.S. (1965). Intermediate States in the Precipitation of Hydroxyapatite, *Nature*, **208**: 365–367.
22. Vogel, G.L., Chow, L.C. and Brown, W.E. (1983). A Microanalytical Procedure for the Determination of Calcium, Phosphate and Fluoride in Enamel Biopsy Samples, *Caries Res.*, **7**: 23–31.
23. Murphy, J. and Riley, J.P. (1962). Single Solution Method for the Determination of Phosphate in Natural Waters, *Ana. Chim. Acta*, **27**: 31–36.
24. Ishiyama, M., Shiga, M., Sasamoto, K., Mizoguchi, H. and He, P.-G. (1993). A New Sulfonated Tetrazolium Salt that Produces a Highly Water-soluble Formazan Dye, *Chem. Pharm. Bull.*, **41**: 1405–1412.
25. Jarcho, M. (1981). Calcium Phosphate Ceramics as Hard Tissue Prosthetics, *Clin. Orthop.*, **157**: 260–268.
26. Nishimura, N., Yamamura, T., Taguchi, Y., Ikenaga, M., Nakamura, T., Kokubo, T. and Yoshihara, S. (1991). A New Bioactive Bone Cement: Its Histological and Mechanical Characterization, *J. Appl. Biomater.*, **2**: 219–229.
27. Kurashina, K., Kurita, H., Hirano, M., de Blieck, J.M.A., Klein, C.P.A.T. and de Groot, K. (1995). Calcium Phosphate Cement: *In Vitro* and *In Vivo* Studies of the α-tricalcium Phosphate - Dicalcium Phosphate Dibasic – Tetracalcium Phosphate Monoxide System, *J. Mater. Sci.: Mater. Med.*, **6**: 340–347.
28. Kurashina, K., Kurita, H., Kotani, A., Klein, C.P.A.T. and de Groot, K. (1997). *In Vivo* Study of Calcium Phosphate Cements: Implantation of an α-tricalcium Phosphate/dicalciuum Phosphate Dibasic/tetracalcium Phosphate Monoxide Cement Paste, *Biomaterials*, **18**: 539–543.

29. Koshino, T., Kubota, W. and Morii, T. (1996). Bone Formation as a Reaction to Hydraulic Hydroxyapatite Thermal Decomposition Product Used as Bone Cement in Rabbits, *Biomaterials*, **16**: 125–128.
30. Miyamoto, Y., Ishikawa, K., Takeuchi, M., Yuasa, M., Kon, M., Nagayama, M. and Asaoka, K. (1996). Non-decay Type Fast-setting Calcium Phosphate Cement: Setting Behavior in Calf Serum and its Tissue Response, *Biomaterials*, **17**: 1429–1435.
31. Eanes, E.D. (1998). *Amorphous Calcium Phosphate: Thermodynamic and Kinetic Considerations. Calcium Phosphates in Biological and Industrial Systems*. Kluwer Academic Publ., Boston, MA, pp. 21–39.
32. Skrtic, D., Antonucci, J.M., Eanes, E.D. and Brunworth, R.T. (2002). Silica- and Zirconia-hybridized Amorphous Calcium Phosphate. Effect on Transformation to Hydroxyapatite, *J. Biomed. Mater. Res.*, **59**(4): 597–604.
33. Yamaguchi, T., Chattopadhyay, N., Kifor, O., Butters Jr, R.R., Sugimoto, T. and Brown, E.M. (1998). Mouse Osteoblastic Cell Line (MC3T3-E1) Expresses Extracellular Calcium (Ca^{2+})-sensing Receptor and Its Antagonists Stimulate Chemotaxis and Proliferation of M3CT3-E1 Cells, *J. Bone Miner. Res.*, **13**: 1530–1538.
34. Hynos, I., Edgar, A.J., Buttery, L.D.K., Hench, L.L. and Polak, J.M. (2001). Gene-expression Profiling of Human Osteoblasts Following Treatment with the Ionic Products of Bioglass© 45S5 Dissolution, *J. Biomed. Mater. Res.*, **55**: 151–157.
35. Beck Jr, G.R., Zerler, B. and Moran, E. (2000). Phosphate is a Specific Signal for Induction of Osteopontin Gene Expression, *PNAS*, **97**: 8452–8357.
36. Keeting, P.E., Oursler, M.J., Wiegand, K.E., Bonde, S.K., Spelsberg, T.C. and Riggs, B.L. (1992). Zeolite A Increases Proliferation, Differentiation and Transforming Growth Factor Beta Production in Normal Adult Human Osteoblast-like Cells *In Vitro*, *J. Bone Miner. Res.*, **7**: 1281–1289.
37. Spahl, W., Budzikiewicz, H. and Geurtsen, W. (1998). Determination of Leachable Components from Four Commercial Dental Composites by Gas and Liquid Chromatography/mass Spectrometry, *J. Dent.*, **26**: 137–145.
38. Pelka, M., Distle, R.W. and Petshelt, A. (1999). Elution Parameters and HPLC-detection of Single Components from Resin Composite, *Clin. Oral Invest.*, **3**: 194–200.
39. Schweikl, H., Schmalz, G. and Spruss, T. (2001). The Induction of Micronuclei *In Vitro* by Unpolymerized Resin Monomers, *J. Dent. Res.*, **80**(7): 1615–1620.
40. Ratanasathien, S., Wataha, J.C., Hanks, C.T. and Denison, J.B. (1995). Cytotoxic Interactive Effects of Dentin Bonding Components on Mouse Fibroblasts, *J. Dent. Res.*, **74**: 1602–1606.