New acrylic bone cements conjugated to vitamin E: Curing parameters, properties, and biocompatibility

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Abstract: Acrylic bone cement formulations with antioxidant character were prepared by incorporation of a methacrylic monomer derived from vitamin E (MVE). Increasing concentrations of this monomer provided decreasing peak temperature values, ranging from 62 to 36°C, and increasing setting time with values between 17 and 25 min. Mechanical properties were evaluated by compression and tension tests. Compressive strength of the new formulations were superior to 70 MPa in all cases. The cement containing 25 wt % MVE, however, showed a significant decrease in tensile properties. Biocompatibility of the new formulations was studied *in vitro*. The analysis of the effect of leachables from cements into the media showed continued cell proliferation and cell viability with a significant increase for the cement containing 15 wt % MVE. This formulation also showed a significant increase in cellular proliferation over a period of 7 days as indicated by the Alamar Blue test. The cells were able to differentiate and express phenotypical markers in presence of all materials. A significant increase in alkaline phosphatase activity was observed on the cements prepared in presence of 15–25 wt % MVE compared with PMMA. Morphological assessment showed that the human osteoblast (HOB) cells were able to adhere, retain their morphology, and proliferate on all the cements. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 62: 299–307, 2002

Key words: acrylic bone cements; vitamin E derivative; biocompatibility; *in vitro* testing; biological evaluation

INTRODUCTION

Acrylic bone cements, widely used in orthopedics for the fixation of joint prostheses, are mostly made by *in situ* polymerization of methyl methacrylate (MMA) in presence of polymethyl methacrylate (PMMA) powder.[1] The polymerization reaction is initiated by a redox system with benzoyl peroxide (BPO) as the initiator and a tertiary aromatic amine (*N,N*-dimethyl-4-toluidine) as the activator. The initiator is decomposed by the activator at room temperature to provide free radicals, which act on the monomer molecules to give rise to the polymerized matrix. The disappearance of free radicals from the cement once the polymerization reaction has finished is a process that lasted several weeks[2,3] and could have adverse cytotoxic effects. Free radicals have been shown to be implicated in many diseases because of their reactivity toward cells and tissues.[4]

Cemented hip prostheses still experience a number of failures in the long term, and the loosening phenomena at the cement/bone interface may be due to resorption induced by cytotoxic or inflammatory reactions.[5] The biocompatibility of commercial formulations of acrylic bone cements has been evaluated by different *in vitro* experiments.[6–9] Although the response was benign as expected because of the clinical use of these formulations for many years, the results indicated inflammatory potential[6] genotoxic effects by PMMA or its ingredients.[7–9] Other studies stated that the initial amount of BPO seems to be proportional to the toxic effect on the cells, and this toxicity could be mediated by free radicals.[10]

Vitamin E is a natural biological antioxidant, which prevents peroxides from accumulating and protects cells from damaging effects of free radicals. Vitamin E also ensures the stability and integrity of biological membranes.[11] It has been demonstrated that vitamin E protects against cellular lipid per oxidation in cartilage to sustain normal bone growth and modeling,[12] and

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findings from animal experiments argue for an osteoprotective effect of vitamin E.[13] Vitamin E has been described[14] as an anti-inflammatory agent, although studies over prolonged periods of time are needed to evaluate this effect. The biological antioxidant capability of vitamin E has been investigated *in vivo* by intraperitoneal injection of vitamin E in rats, and it has been concluded that vitamin E seems to play an important role as an antioxidant and as an anticarcinogen.[15]

This article reports on the formulation of acrylic bone cements with antioxidant character with the aim of diminishing the adverse effects of the free radicals still remaining once the cement has set.[3] The experimental cements were formulated by substituting different proportions of the MMA monomer by a new methacrylic derivative of vitamin E[16] in the liquid phase. Curing parameters of the cements were determined, and mechanical properties were evaluated by means of tension and compression testing. In addition, cytotoxicity was investigated using several methods that monitor different aspects of cellular activity. MTT assay was used to measure cell metabolic function. This test is dependent on the intact activity of a mitochondrial enzyme, succinate dehydrogenase, which may be impaired after exposure of cells to toxic species.[17] Cell growth and proliferation was assessed using Alamar Blue and total DNA. Alamar Blue assay is based on a redox reaction by cells metabolic activity resulting in a chemical reduction of the surrounding environment.[18] Total DNA was measured using the Hoescht method.[19] Biochemical detection of alkaline phosphatase (ALP) activity was used as an indicator of osteoblast phenotype.[20] Scanning electron microscopy (SEM) and fluorescence microscopy were performed in order to determine the short term behavior of the HOBs on the materials.

MATERIALS AND METHODS

Materials

Vitamin E methacrylate (MVE) was synthesized as described in a previous article.[16] PMMA commercial beads (33 μm of average diameter) were supplied by Industrias Quirúrgicas de Levante and previously characterized.[21] MMA monomer stabilized with 100 ppm of monomethylether of hydroquinone was supplied by Merck and used as received without further purification. BPO, supplied by Merck, was used after crystallization from ethanol. 4-*N,N*-dimethylamino benzyl alcohol (DMOH), was synthesized as described previously.[22] Tissue culture media, additives, and Thermanox control discs were supplied by Life Technologies Ltd. (Paisley, United Kingdom) and plasticware by Becton-Dickinson (Oxford, United Kingdom). Trypsin, phophate-buffered saline (PBS), ascorbate, and MTT were supplied by Sigma Chemical Co. (Poole, United Kingdom). All other reagents were supplied by Merck Ltd. (Poole, United Kingdom).

Preparation and characterization of acrylic bone cements

The experimental bone cements were formulated by adding the liquid component to the solid component at room temperature in a solid:liquid ratio of 1.8:1. The solid component consisted of PMMA beads blended with the initiator BPO in a concentration of 1.5 wt %. The liquid phase consisted of mixtures of MMA/MVE in different proportions and 1 wt % of DMOH as a low toxicity activator.[23] The concentrations of MVE in the liquid phase were 10, 15, 20, and 25 wt % in different experiments.

Curing parameters

Setting time and maximum temperature of the reacting mass were determined according to ASTM standard specification.[24] The exothermic polymerization temperature was recorded at 25°C. The reacting mass was introduced in a cylindrical Teflon mold 10 mm in diameter and 15 mm high, equipped with a thermocouple connected to a high sensitivity temperature recorder, and positioned in the center of the mold at a height of 3.0 mm in the internal cavity. The design of the mold used in all the experiments has been published previously.[21] The advantages of using this mold are that it needs less than half the quantity of material and it provides isothermal control by means of a thermostatically controlled box. In addition, the size and shape of the mold reproduces the intrafemoral cavity. The correlation of this mold with that described in the standard ASTM (F451) was analyzed by means of parallel experiments, and the deviation of the temperature for both methods was less than ±2°C.

Residual monomer content

Residual monomer content was determined by means of proton NMR spectroscopy with a Varian XL300 spectrometer. Three samples of each type were dissolved in deuterated chloroform (5 % w/v) using tetramethylsilane as internal standard. All the specimens were kept for 7 days in air before the analysis. This criterion has been applied according to the rules adopted with other international laboratories and by taking into consideration that after 7 days, it can be considered that the polymerization reaction reaches the maximum conversion.[25]

Thermal properties of the cements

Glass transition temperatures (T_g) were measured by differential scanning calorimetry with a Perkin Elmer DSC7 interfaced to a thermal analysis data system TAC 7/DX. The dry samples were prepared in the form of thin films (15–20

mg) placed in aluminium pans and heated from 50 to 200°C at a constant rate of 10°C/min. T_g was taken as the midpoint of the heat capacity transition.

Molecular weight distributions of the cements

Number and weight average molecular weight and polydispersity were determined by size exclusion chromatography (SEC; Waters 510 with a refractive index detector series 200). A set of 10^4, 10^3, and 500 Å PL-gel columns conditioned at 25°C were used to elute the samples of 10 mg/mL concentration at 1 mL/min HPLC-grade chloroform flow rate. Calibration of SEC was carried out with monodisperse standard polystyrene and PMMA samples obtained from Polymer Laboratories.

Mechanical properties

Tension and compression testing were carried out at room temperature in an Instron universal testing machine. A crosshead speed of 20 mm/min was used in the compression testing according to ASTM standard specification,[24] whereas a load cell of 0.5 kN and a crosshead speed of 1 mm/min was used in the tension testing.[26] Specimens were tested after storage in air for 7 days. One-way ANOVA was performed for mechanical properties results at 0.05 level of significance ($p = 0.05$).

Specimens and *in vitro* cell culture for biocompatibility experiments

Rectangular shaped specimens (approximately 10 × 10 × 2 mm) from acrylic bone cements of PMMA and those containing 10 wt % MVE, 15 wt % MVE, and 25 wt % MVE were used for direct and indirect biocompatibility experiments. All the specimens were sterilized by gamma irradiation at a dose of 2.5 Mrad (Swann Morton, United Kingdom) using standard procedures for medical devices. The negative control was tissue culture plastic [Thermanox (TMX) an international standard] and the positive control (toxic agent) was polyvinylchloride (PVC). Primary human osteoblast (HOB) cells were isolated from trabecular bone fragments obtained from femoral heads of patients undergoing hip replacement surgery[27] and cultured at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal calf serum (FCS), 1% nonessential amino acid (NEAA), 0.02*M* HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (all Life Technologies) and 150 μg/mL ascorbate. The culture medium was changed every 3 days.

Evaluation of cytotoxicity of "leachables" from the cured materials

MTT assay

TMX, PVC, and rectangular specimens (110 mg) of experimental cements were set up in 5 mL of DMEM, FCS-free and ascorbate-free (ascorbate interferes with the MTT assay). They were placed on a roller mixer at 37°C, and the medium was removed at different time periods (4 h, 24 h, 48 h, and 7 days) and replaced with other 5 mL of fresh medium. All the extracts were obtained under sterile conditions. HOB cells were seeded at a density of 1×10^4 cells/well in complete medium in a sterile 96-well culture plate and incubated to confluency. The medium was replaced with the corresponding eluted extract (100 μL/well) after the correction of the FCS concentration. The plates were incubated at 37°C in a humidified air with 5% CO_2 for 24 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared in warm PBS and filtered before use. MTT, 10 μL, was added to all wells to give a final concentration of 0.5 mg/mL, and the plates were incubated at 37°C, 5% CO_2 for 4 h. Excess medium and MTT were removed, and 100 μL dimethyl sulphoxide (DMSO) was added to all wells in order to solubilize the MTT taken up by the cells. This was mixed for 10 min, and the absorbance was measured on a Dynatech MR700 plate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Cell culture evaluation

Test specimens and TMX controls for determining cell proliferation (Alamar Blue assay), DNA content, and ALP activity were placed in four sets of 24-well Falcon sterile culture plate. HOB cells were seeded at a density of 2×10^6 cells/mL. On each specimen 50 μL cell suspension was applied with great care to avoid unwanted cell attachment to the surrounding surface of the culture dish and allowed to attach for 1 h before flooding with 1 mL DMEM medium. The cultures were incubated at 37°C in a humidified air with 5% CO_2 for 1, 3, 7, and 14 days. The culture medium was carefully changed at appropriate time intervals to minimize disturbance to the culture conditions.

Alamar Blue assay

Alamar Blue dye (2 mL of 10% Alamar Blue solution in phenol red free DMEM medium) was added to each specimen. After 3 h of incubation, 100 μL (n = 4) of culture medium for each test sample was transferred to a 96-well plate. The fluorescence was measured on a Fluoroskan fluorimeter at an excitation wavelength of 510 nm and an emission wavelength of 590 nm. The cells were then lysed by freeze-thawing the samples. The culture medium was replaced with 1 mL of distilled water, and the samples were frozen at −70°C for 15 min and thawed at 37°C for 20 min three times. The aliquots were stored at −20°C till use.

Measurement of total DNA

A 100-μL aliquot of cell lysate (in duplicate) was mixed with 100 μL Hoescht 33285 (1 μg/mL) in a 96-well plate. DNA standard was prepared with concentrations of 0, 0.31,

0.62, 1.25, 2.50, 5.00, 10.00, and 20.00 μg/mL in saline sodium citrate (SSC) buffer (pH 7) and mixed with Hoescht dye. The fluorescence was measured on a Fluoroskan fluorimeter at an excitation wavelength of 355 nm and an emission wavelength of 450 nm. The DNA content for each test specimen was calculated from the standard DNA curve.

Biochemical measurement of ALP activity

The ALP activity was measured in cell lysate. It was determined using a COBAS-BIO (Roche, Lewes, East Sussex, United Kingdom) centrifugal analyzer. The enzyme ALP cleaves the phosphate group from *p*-nitrophenyl phosphate to produce *p*-nitrophenol, which is yellow at alkaline pH and can be monitored at 405 nm. The analyzer calculates the results using the reaction rate method.

ALP/DNA ratio

Determination of the ALP/DNA ratio is indicative of the amount of ALP activity per cell. The variations caused by the different shape of the test samples can be eliminated using this approach. Both were measured from cell lysate.

Microscopal examination

Scanning Electron Microscopy

The materials were placed in a 24-well plate (in duplicate) and seeded with HOB cells at a density of 1.5×10^5 cells/mL. These were incubated at 37°C in humidified air and 5% CO_2. The cells were fixed with 1.5% glutaraldehyde buffered in 0.1M sodium cacodylate after a 48-h incubation period. The cells were postfixed in 1% osmium tetroxide and 1% tannic acid, then dehydrated through a series of alcohol concentrations (20, 30, 40, 50, 60, and 70), stained in 0.5% uranyl acetate, and then dehydrated further (90, 96, and 100% alcohol). The final dehydration was in hexamethyldisilazane (HMDS), followed by air drying. Once dry, the samples were sputter-coated with gold before examination under a Jeol SEM at an accelerating voltage of 5 keV.

Fluorescence microscope (live/dead assay)

A combination of calcein AM, a nonfluorescent cell-permeant dye that is cleaved by intracellular esterases to fluorescent calcein, and ethidium homodimer, which passes through damaged cell membranes to bind DNA,[28] was used for viability determination. The cells were seeded directly on the bone cements at a cell density of 1.5×10^5 cells/mL for 24 h. The culture media was removed, and 1 μg/mL of ethidium homodimer and calcein AM in supplemented media was added to the cells. These were incubated for 1 h at 37°C. Then the specimens were viewed under a fluorescent microscope with UV illumination (Olympus).

Statistical analysis of biocompatibility test.

The statistical analysis of the cement extracts was made by analysis of variance (ANOVA), and *t* test was used for statistical evaluation of total DNA, Alamar Blue, and ALP results. In all statistical analyses, $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Preparation of acrylic bone cements containing vitamin E methacrylate

The acrylic bone cement currently used in orthopedic surgery are set by a free radical exothermic polymerization process initiated by the reaction between BPO (initiator) and *N,N*-dimethyl-4-toluidine (DMT; activator), which produces benzoate and amine radicals. These radicals then initiate polymerization of the monomer.[29] However, the redox reaction also produces side compounds that are not completely characterized, e.g., the presence of phenolates has been identified by nuclear magnetic resonance.[30] These side products are chemically stable and can leak from the polymer and induce a local inflammatory response.[31] Likewise, more recently, Moreau et al.[3] have reported that release of free radicals from PMMA cements is a long-lasting event that can induce bone cells alterations in their neighborhood. In an attempt to counteract these problems, new formulations with antioxidant character have been developed, which use incorporation of a methacrylic monomer derived from vitamin E. This monomer provides antioxidant characteristics due to the proved efficiency of vitamin E as a free radical "scavenger" in oxidative processes.[32] The modified cements were formulated with the BPO/DMOH initiation system, in which the traditional DMT was substituted by an amine compound of reduced toxicity.[23] The accelerating effect of this activator in the setting of PMMA bone cements has already been demonstrated, providing exotherms of decreasing peak temperature and increasing setting times, although values were within those permitted by the standard specification.[33] The typical solid:liquid ratio of 2:1 was changed to 1.8:1 due to the incorporation of a high-molecular-weight monomer. The variation of this parameter produces an increase in both setting time and peak temperature,[34] although the setting characteristics of the cement are mainly influenced by the chemical composition of the acrylic formulation, which in addition, control the physical and mechanical properties of the cement.[35]

The presence of MVE in the formulations increases the setting and working times respect to the traditional PMMA formulations, but these parameters are

only an orientation and they can be adapted when working with a normal dose of acrylic cement (40 g of solid phase plus 20 mL of liquid phase) if necessary, by increasing 2 or 3° the temperature during the mixing process of both components. An increase in the working time can be beneficial from a practical point of view to favor the implantation of the reacting mass in the bone cavity. Partial replacement of MMA by MVE yielded very interesting formulations, with peak temperature values varying from 62 to 36°C, that is, values rather lower than 56°C, which corresponds to the onset of coagulation of albumin.[36]

Figure 1 shows the time-temperature profiles of several formulations containing the methacrylic derivative of vitamin E, where this effect can be clearly seen. There are numerous references in the literature about the role of exotherm on necrosis of the surrounding bone. The amount of heat released depends on the weight of reacting monomer in the mixture. The reduction of the exotherm of acrylic bone cements by means of the incorporation of monomers with high molecular weight has already been reported.[37,38] A lower exotherm will benefit the biocompatibility of the cement, with the adjacent tissues usually impaired with initial bone necrosis.[39] The presence of MVE modifies noticeably the expected heat of polymerization in Joules per gram, with respect to the pure MMA because the M_{MMA}/M_{MVE} molecular weight ratio is 4.46. However, according to the molecular structure of MVE, and the data reported in Table I, it could be considered a small influence of MVE in the kinetic parameters of the polymerization reaction. The analysis of this point is in progress.

Residual monomer content of the cements modified with the vitamin E-containing monomer was lower than 3 wt % in all formulations, indicating that the polymerization reactions reached conversions similar to those obtained with PMMA bone cements (see values in Table II).

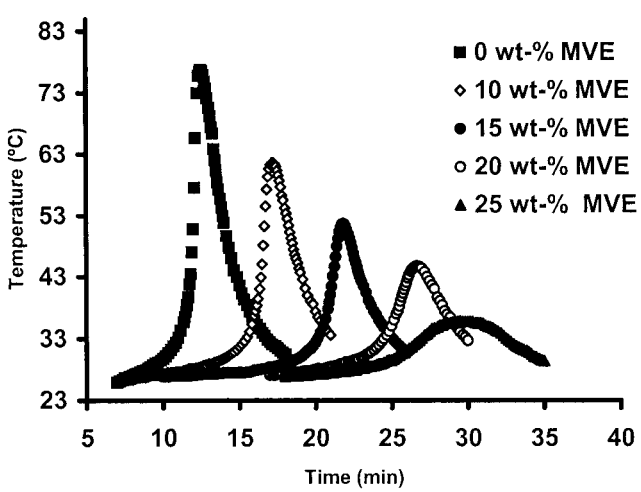

Figure 1. Time-temperature profiles of bone cements formulated with different MMA/MVE ratios in the liquid phase.

TABLE I
Values of Number and Weight Average Molecular Weights (M_n and M_w, respectively), Polydispersity (M_w/M_n), and Glass Transition Temperature (T_g) of Acrylic Bone Cements Modified with Different Amounts of Vitamin E Methacrylate in the Liquid Phase

MVE (wt %)	M_n ($\times 10^{-3}$)	M_w ($\times 10^{-3}$)	M_w/M_n	T_g (°C)
0	82	225	2.7	99
10	70	245	3.5	95
15	68	204	3.0	93
20	60	198	3.3	89
25	64	235	3.7	91
PMMA[a]	64	128	2.0	—

[a]PMMA, commercial PMMA solid phase.

Molecular weight and *Tg*

The values of number and weight average molecular weights, polydispersity, and T_g of cements modified with vitamin E methacrylate are shown in Table I. The molecular weight of the cured cement corresponds to the average of the initial solid PMMA phase plus the polymeric matrix formed by the free radical polymerization process. All the cements present high values of molecular weight and a molecular weight distribution, according to a normal polymerization process. A slight decrease in molecular weight with the content of MVE in the liquid phase was observed; however, changes in the values of this parameter are insufficient as to detect a clear effect of the composition of the liquid phase on the length of the macromolecular chains. On the other hand, values of T_g of the modified cements were comparable to that of PMMA cement, although slightly lower, which can be attributed to the plastizicing effect produced by the long side group incorporated in the monomer derived from vitamin E. However, values of both molecular weights and T_g of the modified cements were in the range of those reported for commercially available cements.[25]

TABLE II
Values of Curing Parameters and Residual Monomer Content for Acrylic Bone Cement Formulations Containing a Methacrylic Derivative of Vitamin E

MVE (wt %)	t_{dough} (min)	$t_{setting}$ (min)[a]	$t_{working}$ (min)[a]	T_{peak} (°C)[a]	R_M (%)[a]
0	4.5	12.2 (0.3)	7.5 (0.3)	77.5 (2.1)	1.8 (0.2)
10	4.0	16.6 (0.8)	12.5 (0.8)	60.3 (1.9)	2.0 (0.0)
15	3.5	20.7 (0.4)	17.5 (0.4)	53.0 (1.1)	1.8 (0.2)
20	3.3	24.6 (0.3)	21.2 (0.3)	46.0 (1.7)	2.6 (0.1)
25	3.0	24.7 (0.7)	22.0 (0.7)	35.8 (0.0)	2.2 (0.2)

[a]Values in parentheses are standard deviation.

TABLE III
Mechanical Properties of Formulations of Acrylic Bone Cement Modified with a Methacrylic Derivative of Vitamin E[a]

MVE (wt %)	σ_c (MPa)[b]	E_c (GPa)[b]	σ_t (MPa)[b]	ϵ (%)[b]	E_t (GPa)[b]
0	100 (10)	1.3 (0.1)	51 (5)	3.1 (0.3)	2.1 (0.1)
10	105 (8)	1.4 (0.1)	49 (5)	2.9 (0.4)	2.1 (0.1)
15	10 (4)	1.4 (0.1)*	46 (3)	2.9 (0.2)	1.9 (0.1)*
20	93 (5)	1.5 (0.1)*	47 (2)	3.3 (0.2)	1.9 (0.1)*
25	98 (7)	1.5 (0.1)*	44 (4)*	2.7 (0.2)*	1.9 (0.1)*

[a]Compressive strength (σ_c), Young's modulus in compression (E_c), tensile strength at break (σ_t), Young's modulus in tension (Et) and strain to failure (ϵ).
[b]Values in parentheses are standard deviation.
*Statistically significant with respect to PMMA.

Mechanical properties

In clinical service static or quasistatic direct compressive and tensile forces are important. In hip joint replacements the cement mantle is subjected to compressive forces between the femoral stem and the bone tube by acting as a shock absorber and a decoupler between the implant and the bone. Also, tensile stresses are experienced in various parts of an arthroplasty, e.g., on the lateral side of a hip implant, due to bending.[1] Then, compression and tension testing of the experimental formulations was carried out, the results of which are summarized in Table III. Young's modulus in compression showed a significant increase when MVE was present in a concentration ranging between 15 and 25 wt %, and compressive strength did not significantly change with the incorporation of up to 25 wt % MVE to the liquid phase. Regarding tensile properties, tensile strength remained unchanged in formulations containing less or 20 wt % MVE, although a decrease in this parameter was observed for the cement containing 25 wt % MVE. The effect of the presence of MVE on the strain to failure followed the same trend as tensile strength, that is, only a significant decrease of this parameter was observed for the cement formulated with the highest concentration of MVE. Young's modulus decreased significantly from a concentration of 15 wt % MVE. In general, it can be said that the values of the mechanical properties in compression and tension are in the range of those reported in the literature for commercial formulations.[1]

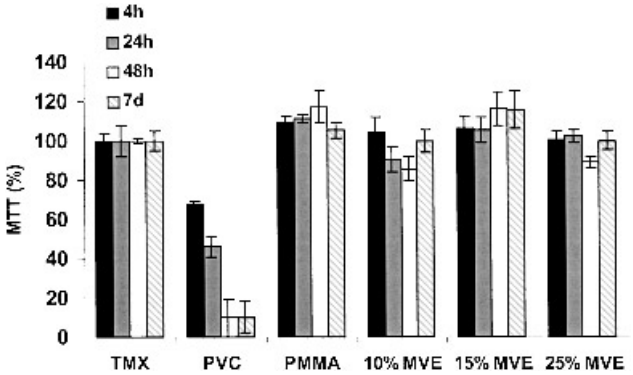

Figure 2. MTT cytotoxicity results for the controls, TMX and PVC, and the experimental bone cements formulated in this work (results are the mean ± standard deviation; $n = 8$).

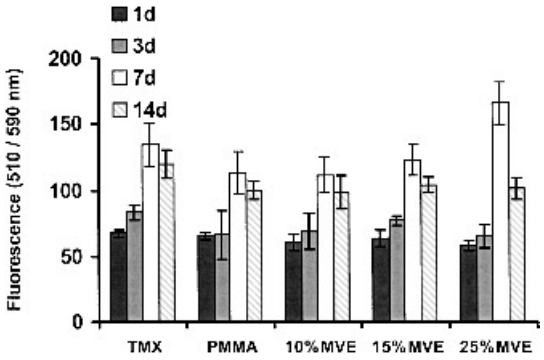

Figure 3. DNA (μL) in cell lysate. HOB cells cultured directly on test materials over a period of 14 days (results are the mean ± standard deviation; $n = 3$).

Evaluation of cytotoxicity of "leachables" from the cured materials

MTT results for the cements formulated in the presence of vitamin E metacrylate are shown in Figure 2. A significant drop in cell viability in the presence of the eluates for the cement containing 10 wt % MVE at 24 and 48 h and for the cement containing 25 wt % MVE at 48 h was obtained; this returned to normal in subsequent elutions, indicating the absence of mitochondrial damage, as a result of "leachable toxics" in any of the cements. The cement prepared with 15 wt % MVE showed a significant increase in cell viability compared with the negative control TMX at any time. This indicates that the bone cement extracts of any of the

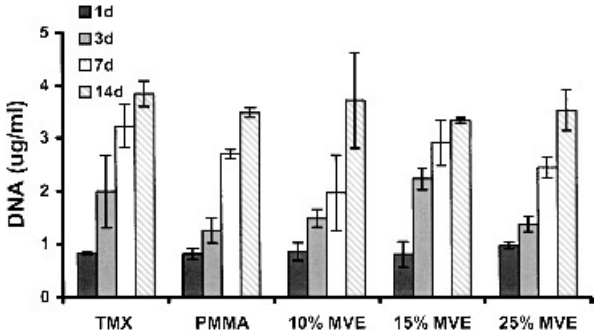

Figure 4. Direct Alamar Blue results for control (TMX) and the experimental cements containing vitamin E methacrylate cultured over a period of 14 days (results are the mean ± standard deviation; $n = 3$).

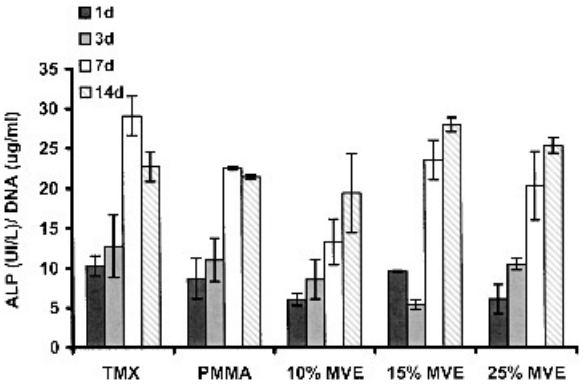

Figure 5. Alkaline phosphatase/DNA activity in HOB cells cultured directly on test materials over a period of 14 days (results are the mean ± standard deviation; $n = 3$).

formulations did not produce a negative effect on mitochondrial cell activity during the assay and as such were not considered toxic.

Cell growth, proliferation, and differentiation

Cell growth and proliferation were assessed by direct Alamar Blue and total DNA. Alamar Blue results for the control and the experimental cements are plotted in Figure 3, showing that, in all cases, the HOB proliferated between day 1 and day 7 and from then on, the cells kept on dividing, forming multilayers but a much slower rate. Once proliferation begins to decline, cells are able to differentiate, and this is indicated by the increase in ALP activity. This activity acts as a signal for the subsequent production of the proteins leading to mineralization.[40] A significant increase in the proliferation in day 7 was obtained for the cement formulated with 25 wt % MVE compared with PMMA cement. Cell growth and cell proliferation as assessed by total DNA is shown in Figure 4. An increase in total cellular DNA with time was observed on all the materials over the 14-day culture period. No significant differences were observed in the cell proliferation on the cements prepared with vitamin E methacrylate compared with PMMA, except for the cement containing 15 wt % MVE on which DNA content showed a significant increase at day 3. ALP levels were normalized for DNA, and results are plotted in Figure 5. ALP activity was seen to follow normal behavior of cell differentiation[40] for all the formulations. A significant increase in ALP activity was observed for

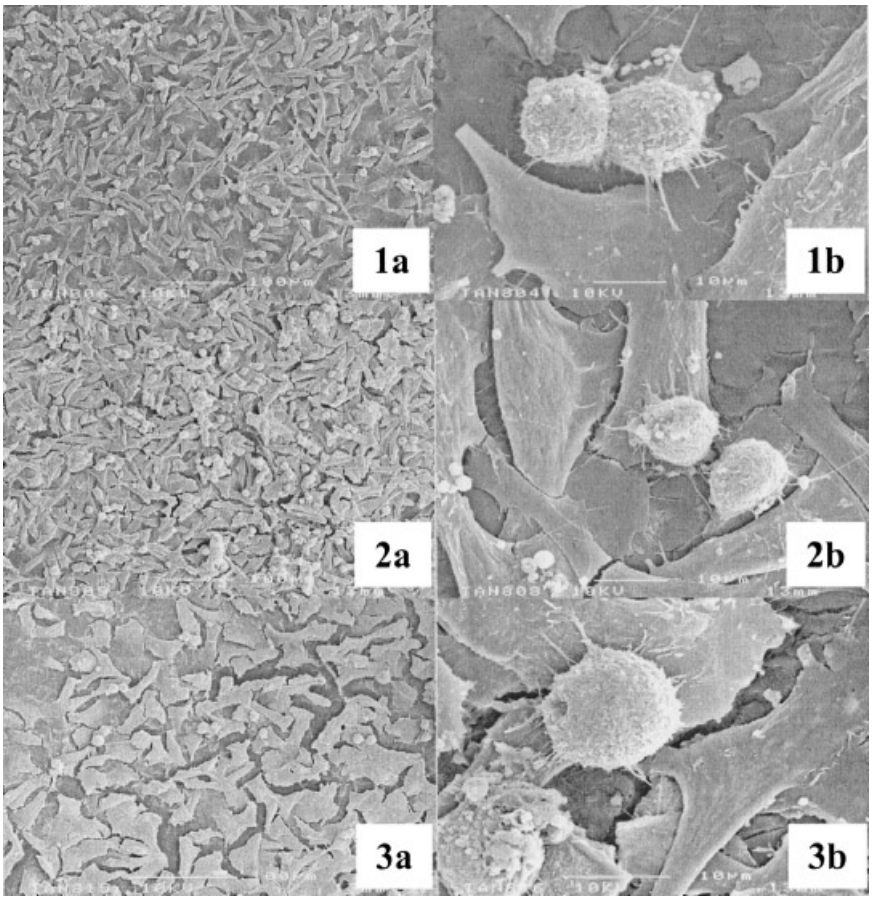

Figure 6. SEM images of HOB cell colonization on the bone cement specimens at ×90 (a) and ×430 (b) magnification. (1) PMMA; (2) 15 wt % MVE; and (3) 25 wt % MVE.

the cements prepared in the presence of 15 and 25 wt % MVE compared with PMMA cement in a period of 14 days.

Microscopy study

The biocompatibility of biomaterials is very closely related to cell behavior in contact with them, and particularly cell adhesion to their surface. Attachment, adhesion, and spreading are part of the first phase of cell/material interactions, and the quality of these processes will influence the cell's capacity to proliferate and to differentiate itself on contact with the implant.[41] Figure 6 shows some photomicrographs of the HOB cells colonization on cements containing vitamin E along with that on PMMA. All cured cements showed good biocompatibility, and no adverse effect on the cells were observed. Cells were able to adhere and proliferate on the experimental cements, indicating that there was no direct toxic effects and cellular metabolism was normal. The cells retained osteoblast morphology and were seen to form a layer of cells with numerous dividing cells on all materials. Cell adhesion and proliferation is particularly important in materials designed to be integrated into host tissues as in the case of osseointegration. In addition, dead and live assay demonstrated normal cell activity (at least as good as TMX). No dead cells were observed on top of the materials.

CONCLUSIONS

New acrylic bone cements have been prepared with incorporation of vitamin E methacrylate, the optimum concentration of this monomer being in the range 15–25 wt % with respect to the liquid phase. The presence of 15–25 wt % MVE provided a reduction in peak temperature from 25 to 40°C, and the finished materials presented acceptable mechanical properties. Likewise, the cements containing 15 and 25 wt % MVE provided the best biocompatibility results. No toxic leachables were released to the medium. Cell viability, proliferation, and differentiation indicated that the presence of the vitamin E-containing monomer has contributed to cytocompatibility. In addition, the implantation of such formulations *in vivo* will contribute to the biological stabilization of the prostheses, because of the strong reduction of the peak temperature during the curing of the cement.

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