The effect of varying percentage hydroxyapatite in poly(ethylmethacrylate) bone cement on human osteoblast-like cells

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Poly(ethylmethacrylate) (PEMA) bone cement has been developed, and the cements mechanical properties are improved by the incorporation of particulate fillers, such as hydroxyapatite (HA). In this *in vitro* study, human osteoblast-like (HOB) cells were used to examine the effect on cellular behavior of the addition of HA to PEMA using a plain PEMA control. Thymidine uptake (^{3}H-TdR) and total DNA were used to assess cell growth and proliferation. Confocal laser scanning microscopy (CLSM) was used to study focal contacts and actin cytoskeletal organisation. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to assess cell morphology and cellular ultrastucture. The early time points showed preferential anchorage to the HA exposed on the cement surface, but no difference in adhesion or proliferation. These results have been attributed to increases in residual monomer with HA incorporation, as shown by proton nuclear magnetic resonance (H^1-NMR) spectra.

1. Introduction

The long-term success of orthopaedic implants depends on successful early implant fixation and stability. Walker *et al*. [1] showed that the rate of migration of total hip replacement (THR) prosthesis after 2 years is predictive of the long-term outcome of THR arthroplasty.

The early days of joint replacement arthroplasty were noted for the high rate of loosening mainly due to poor implant fixation. The advent of polymethylmethacrylate (PMMA) bone cement in the 1960s, developed by Charnley [2], has revolutionised the success rate of joint replacement arthroplasty in contemporary orthopaedics. PMMA, a self-curing acrylic polymer, and grouting agent, firmly fixes prosthetic components to bone [3].

PMMA bone cement has been shown to have a success rate of 90% at 15 years post implantation [4]. Despite the relative success rate of PMMA, it does have some limitations, including, high exothermic temperature of polymerization (67 °C–124 °C) [5], leading to thermal bone necrosis, and chemical necrosis due to leachable monomers [6]. Shrinkage during polymerization also occurs and in addition, it has a much higher modulus compared with adjacent bone leading to interfacial stress formation [7].

The modulus mismatch coupled with the space created by thermal and chemical necrosis, and polymer shrinkage has a potential to create micromotion at the bone–cement interface, which leads to fibrous encapsulation. The fibrous layer leads to further micromotion, in turn generating wear particles. This layer may also act as a conduit for wear particles into the joint space at the bone–cement interface. These factors culminate in aseptic loosening (concurrent with the micromotion theory of aseptic loosening) [8]. PMMA cement is described as brittle, with low material toughness and poor fatigue properties associated with porosity [9].

These disadvantages coupled with increasing life expectancy of the general population and the increasing

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need for joint replacement arthroplasty in younger patients, has lead to the development of a novel bone cement, polyethylmethacrylate using *n*-butylmethacrylate as a monomer (PEMA/*n*-BMA) [10].

PEMA/*n*-BMA has lower exotherm 50–55 °C [11], less polymer shrinkage [12], and 20% less extractable monomer compared with PMMA [13]. It has lower modulus and higher ductility, which confer greater axial movement and better proximal load transfer [14]. PEMA/*n*-BMA with 30% by weight HA was treated with 3-trimethoxysilypropyl methacrylate, a silane coupling agent, to improve PEMA-HA bonding, resulting in an increase in tensile strength and better fatigue properties [15]. HA as a component of a bone cement composite confers bioactivity to the material. The surface of synthetic HA is biologically active leading to the formation of physiological bonds with bone. Various researchers have reported evidence of direct HA-bone bonding *in vivo* and increased biological response *in vitro* with various materials [16–19].

Successful cell attachment is a pre-requisite to direct bone bonding with materials. This is, however, influenced by a number of factors. The ability of the cells to attach to the materials surface depends on the extracellular matrix (ECM) proteins adsorbed onto the material surface; integrin receptor extracellular domains, presented by the cells, interact with this protein overlayer. The integrins interact with amino acid motifs such as the RGD (arginine, glycine and aspartate) sequence found within the protein structures (fibronectin, vitronectin, collagen, etc) [20]. Other factors affecting adhesion include material topography, described as the morphology of the material surface [21, 22].

At the site of integrin attachment to the ECM deposited on the material substrate, focal adhesions are formed [24]. Adhesion plaques are associated with a complex network of cytoskeletal elements linked directly or indirectly with the integrin intracellular domains. The main associated cytoskeletal elements include actin microfilaments, tubulin intermediate filaments, and vimentin microtubules.

The aim of this *in vitro* study was to assess the effect of addition to PEMA/*n*-BMA cement on osteoblast behavior. Human osteoblast-like cells (HOBs) were used as they are representative of the cell type the material would encounter *in vivo* [23]. Cell attachment was studied using confocal laser scanning microscopy (CLSM) to observe focal adhesion points and actin cytoskeletal organization. Tritiated thymidine [^{3}H- TdR] and total DNA were used to assess subsequent cell proliferation. Cell morphology was observed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

2. Materials and methods

2.1. Materials

Disks of 1.2 cm PEMA/*n*BMA [15] cements were prepared by addition of the monomer to the polymer and stirred, until fully wetted, under controlled temperature condition (22 ± 2 °C). HA was added by replacing an equal weight of polymer powder prior to the addition of the monomer. The overall HA content was a volume fraction of 10%, equivalent to 30% by weight. The prepared disks were sterilized by gamma irradiation at a dose of 2.5 Mrad (Swann Morton, UK) using standard procedures for medical devices. Proton nuclear magnetic resonance spectroscopy (H^1-NMR) was performed on the plain PEMA and HA filled PEMA by Queen Mary University of London Chemistry Services (Bruker AMX 600) to allow quantification of unreacted *n*BMA monomer.

2.2. *In vitro* cell culture

HOB cells were isolated from the femoral head of a patient undergoing total joint replacement. Trabecular bone fragments were dissected from the femoral head and washed several times in phosphate buffered saline (PBS), followed by a final wash in complete medium (Dulbecco's modified eagles medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, L-ascorbic acid (150 g/ml) 0.02 M L-glutamine, 0.01 M HEPES, 100 units/ml penicillin and 100 µg/ml streptomycin). The bone chips were further chopped with scalpel blades, and incubated in complete medium at 37 °C, 5% CO_2 in a humid atmosphere. Once an osteoid stem of cells transferring from the fragments to the culture plastic was observed, the chips were transferred to a collagenase (100 U/ml) and trypsin (300 U/ml) in PBS (0.01 M HEPES buffered) solution. The bone was digested on a roller at 37 °C for 20 min. The supernatant was centrifuged (200 rpm, 18 °C, 5 min) and a cell pellet was obtained. The pellet was resuspended in fresh medium (5 ml) and plated into a 25 ml tissue culture flask. The HOBs were characterized by measurement of alkaline phosphatase (ALP) (biochemical and histochemical), osteocalcin, procollagen type I, and response to parathyroid hormone (measurement of cAMP) [23]. For this report, HOB cells were cultured on the test materials and control Thermanox (TMX, Life Technologies) at 1.5×10^6 cells cm^{-2} for 1, 3, 7, 14, and 28 days under conditions described in a previous study [25].

2.3. Cell growth and proliferation

Cell growth and proliferation were assessed using total DNA and tritiated thymidine (^{3}H-TdR) incorporation, in each case $n = 5$ replicates. These methods have been described in a previous study [25].

2.4. Immunofluorescence of vinculin and actin

After 2 and 3 days of culture, the cells on the test materials ($n = 3$) were fixed in 4% formaldehyde/PBS, with 1% sucrose at 37 °C for 15 min. When fixed, the samples were washed with PBS, and a permeabilizing buffer (10.3 g sucrose, 0.292 g NaCl, 0.06 g $MgCl_2$, 0.476 g Hepes buffer, 0.5 ml Triton X, in 100 ml water, pH 7.2) added at 4 °C for 5 min. The samples were then incubated at 37 °C for 5 mins in 1% BSA/PBS, followed by the addition of anti-vinculin primary antibody (1 : 100 in 1% BSA/PBS, hVin1 monoclonal anti-human raised in mouse (IgG1), Sigma, Poole, UK) for 1 h (37 °C). Simultaneously, FITC conjugated phalloidin was added

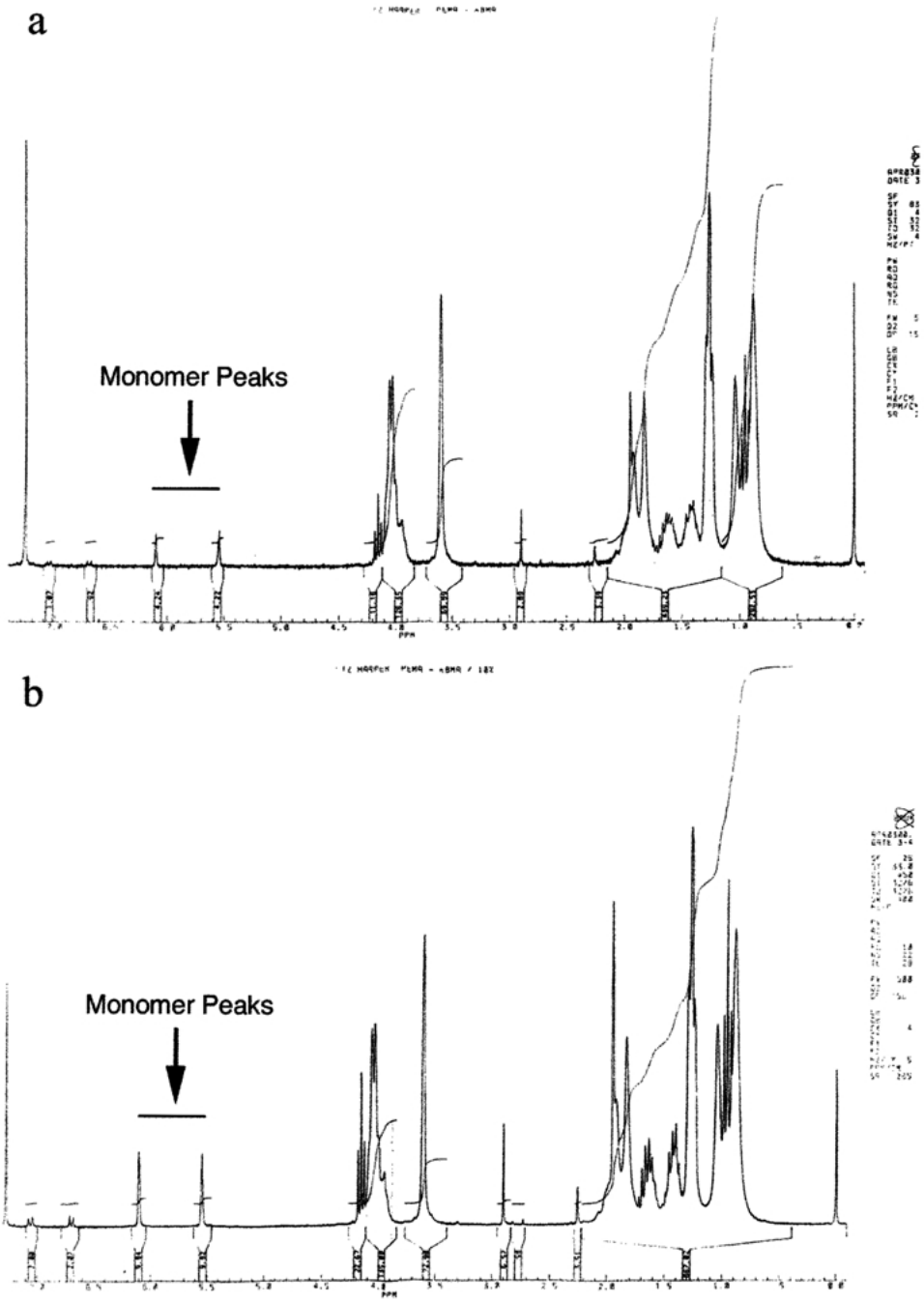

Figure 1 H^1-NMR spectra for PEMA (a) and PEMA/10% vol. HA (b). The traces show increased monomer in (b).

for the duration of this incubation (1 : 100 in 1% BSA/PBS, Sigma, Poole, UK). The samples were next washed in 0.5% Tween 20/PBS (5 min × 3). A secondary, biotin conjugated antibody, (1 : 50 in 1% BSA/PBS, monoclonal horse anti-mouse (IgG), Vector Laboratories, Peterborough, UK) was added for 1 h (37 °C) followed by washing. A Texas red conjugated streptavidin third layer was added (1 : 50 in 1% BSA/PBS, Vector Laboratories, Peterborough, UK) at 4 °C for 30 min, and given a final wash. Finally, the samples were stained for DNA with DAPI at 1 μg/ml (10 min) before viewing by confocal laser-scanning microscope (CLSM, Noran).

2.5. Transmission electron microscopy

HOB cells were seeded onto the materials ($n = 3$, 1×10^6 cells ml^{-1}) and incubated. The cells were fixed with 1.5% gluteraldehyde buffered in 0.1 M sodium cacodylate after 1.5 months in culture. Cells were post-fixed in 1% osmium tetroxide, dehydrated in 70%, 90%, 96%, 100% (sodium sulfate dried) alcohol. Once dehydrated the samples were resin embedded (Spurr's resin [26]) and polymerized at 70 °C for 18 h. Ultra-thin sections were cut and uranyl acetate and lead citrate stained for ultrastructure. The samples were viewed by Phillips CM12 TEM.

2.6. Scanning electron microscopy

Materials were seeded with HOB cells at a density of 1.5×10^4 cells ml^{-1}, which were incubated at 37 °C in humidified air and 5% CO_2. Cells were fixed with 1.5% gluteraldehyde buffered in 0.1 M sodium cacodylate after a 48-h incubation period. Cells were post-fixed in 1%

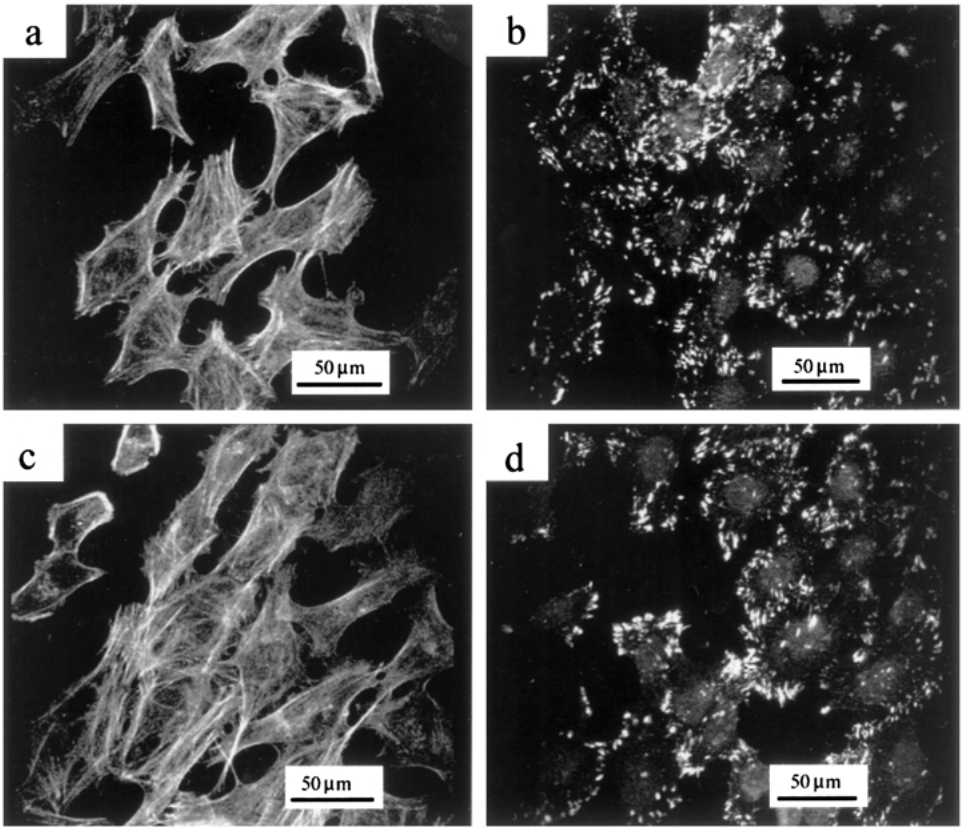

Figure 2 CLSM images for vinculin and actin. No differences in actin cytoskeleton were observed between PEMA (a) and PEMA/10% vol. HA (c). No differences in focal contact formation were observed between PEMA (b) and PEMA/10% vol. HA (d).

osmium tetroxide and 1% tannic acid, then dehydrated through a series of alcohol concentrations (20%, 30%, 40%, 50%, 60%, 70%), stained in 0.5% uranyl acetate (in 70% alcohol), then dehydrated further (90%, 96%, 100% alcohol). The final air-drying was in hexamethyl-disilazane. Once dry, the samples were gold/palladium sputter coated before examination under a JEOL Winsem 3500 SEM.

2.7. Statistics

A multiple comparison statistics test, Tukey Kramer honestly significant difference, was used for statistical analysis using SPSS (a windows based software).

3. Results

3.1. H^1-NMR

Fig. 1 shows the H^1-NMR traces for plain PEMA (a), and PEMA/10% vol. HA (b). The figure shows double the amount of unreacted monomer present in PEMA/10% vol. HA.

3.2. Cell attachment

The CLSM images show normal polygonal osteoblast morphologies were supported on the PEMA and the PEMA/HA test composite (Fig. 2). Actin staining showed mature stress fibers were apparent through the cell bodies (Fig. 2 (a) and (c)), this suggests that the cells were spreading flat on to the plain PEMA and PEMA/HA surfaces. Immunolabeling of vinculin showed many focal contacts had been formed in cells on both the PEMA and PEMA with 10% vol. HA (Fig. 2(b) and (d)). It is noted, however, that there were no distinguishable differences between the plain PEMA and PEMA/HA morphology and cytoskeleton observed.

3.3. Cell morphology

SEM morphological evaluation, while showing no differences in cell area and spreading between the plain PEMA and test PEMA/HA, did show filopodia from the HOB cells anchoring to exposed HA on the PEMA/HA composite surfaces (Fig. 3).

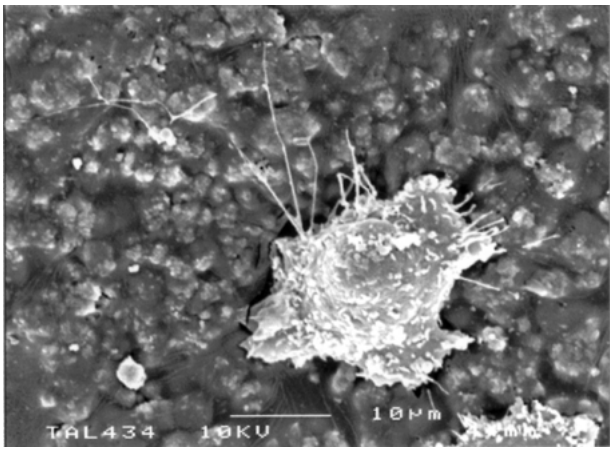

Figure 3 SEM image of a HOB cell cultured on PEMA/10% vol. HA. Note the preferential anchorage of filopodia to exposed HA.

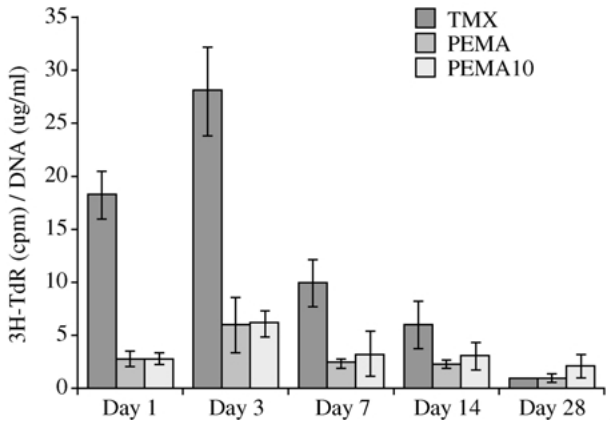

Figure 4 Graph for ^{3}H-TdR incorporation normalized to total DNA. The results show increased proliferation on the PEMA/10% vol. HA composite compared to plain PEMA. This difference is not, however, statistically significant at any time point (results are the mean $\pm$ s.d., $n = 5$).

3.4. Cell growth and proliferation

The thymidine uptake per DNA showed marginal increase in growth and proliferation of HOB cells on PEMA with 10% by vol. HA compared with plain PEMA. This difference, however, was not statistically significant (Fig. 4). A normal growth pattern was noted on the TMX and test substrates, with highest levels of proliferation observed on days 1 and 3. As is normal with HOB cultures, proliferation slowed as the cells became more dense. It is noted that at days 1, 3 and 7 the proliferation on the TMX negative cytotoxicity control was significantly higher than for the test materials, this is not denoted on the graph as the focus is differences between PEMA and PEMA/HA.

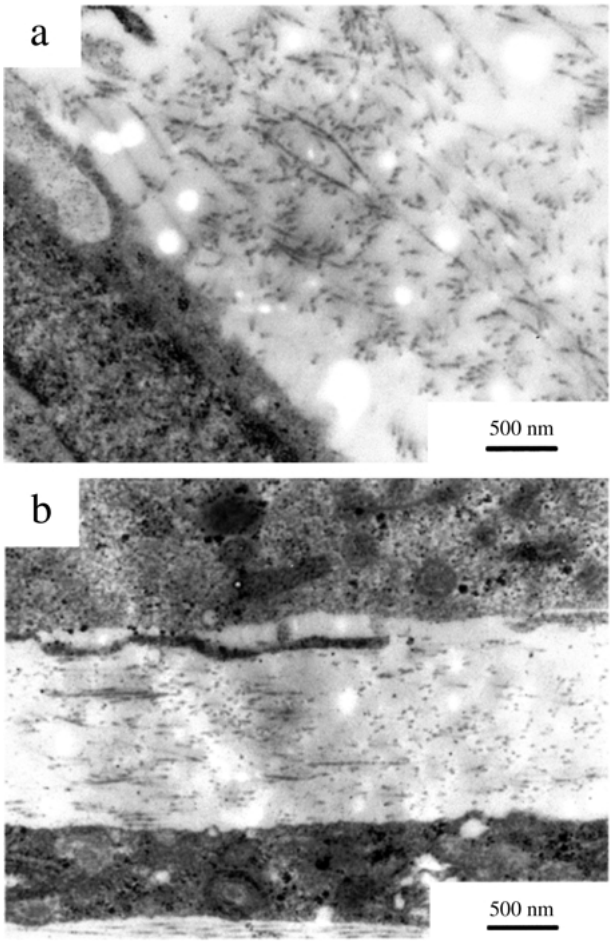

Figure 5 TEM images of HOBs cultured on the test materials for 42 days. The images show extracellular matrix between the HOB cell layers after 1.5 months of culture. Plain PEMA (a) and PEMA/10% vol. HA (b).

3.5. Cell ultrastructure

TEM of the HOB cells showed the formation of matrix in between confluent cell layers (Fig. 5). Both materials supported confluent cells, which were 3–4 layers deep after 1.5 months of culture. HOBs on both the PEMA and the PEMA/HA had produced extensive matrix, demonstrating that both materials supported cell growth.

4. Discussion

Preferential anchorage of HOB cells to HA has been previously described [19, 25], and indicates that exposed HA recruits proteins from the extracellular matrix necessary for HOB adhesion [21, 22, 27]. Additionally, topography has been reported as being important for cell attachment [28]. The presentation of HA in cements is thought to be conducive to cell anchorage, as it is often proud of the polymer surface [29]. It would, therefore, be expected from this that increased numbers of focal contacts would be seen. The CLSM micrographs, however, show no differences in either focal contacts or cytoskeletal organization. Similar cell morphology was observed on the PEMA/HA composite, compared to control, even though preferential anchorage was noted. This observation shows the polymer may have adversely affected cell adhesion, thus explaining why despite the HOBs sensing the presence of HA, the number of focal contacts did not increase.

Organization of actin cytoskeleton is important in signal transduction [30, 31]. In this study, there was no visible difference in actin organization, thus, little difference in proliferation would be expected. The results for thymidine incorporation, which showed that there was a slight, but not statistically significant, increase in proliferation on the HA incorporated cement, tie in with these cytoskeletal observations.

More long-term TEM study, again, showed negligible differences between matrix produced by HOBs on PEMA and PEMA/HA. Production of matrix by osteoblasts is a pre-requisite for bone mineral formation [32]. Thus, it appears that both the plain cement, and the HA reinforced cement are supporting the growth of nascent tissue directly onto the material surfaces.

The results show that despite the preferential anchorage to HA observed with the PEMA/HA composite, any other changes in cell response were negligible, and were certainly not as notable as would have been expected (from studies with other polymers such as HAPEX™ [33, 34] and PMMA [35]).

This result may be due to the increased amount of unreacted monomer observed with HA addition. The formation of PEMA bone cement requires PEMA : n-BMA in a 2 : 1 weight ratio. When HA is added, it

substitutes for PEMA, effectively altering this ratio. This appears to lead to increased levels of unreacted monomer that may have cytotoxic effects. In order to correct this, extra initiator was added in the form of lucidol (a 50% by weight benzoyl peroxide). It is, however, noted that the initiator itself can also present problems with cytotoxicity, thus addition of extra initiator can add to the toxicity of the cured cement mass [36, 37].

5. Conclusions

This investigation has shown that while the cells can sense the presence of HA in the cement, the increased levels of monomer detected by H^1-NMR have cytotoxic effects. This leads to no measurable differences being observed. PEMA itself, has recently been shown to have significantly increased biocompatability compared to PMMA [38], and this study gives more baseline *in vitro* information on plain PEMA, most notably the TEM images showing good matrix formation. Thus, a more full picture of osteoblast response to the cement is being developed.

If the bioactive properties of HA could be imparted upon PEMA, this would be a great advantage as HA incorporation also gives improved mechanical properties [15]. The development of a cement with increased mechanical and biological properties would be desirable, but this study shows the importance of developing the setting conditions to get an optimized curing.

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