Enhanced HAPEX topography: Comparison of osteoblast response to established cement

M. J.DALBY[1]*, W. BONFIELD[2]†, L. DI SILVIO[1]
[1]Institute of Orthopaedics, IRC in Biomedical Materials, Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middlesex, HA7 4LP, UK
[2]IRC in Biomedical Materials, Queen Mary and Westfield College, Mile End Road, London, E1 4NS, UK
E-mail: m.dalby@bio.gla.ac.uk

The use of poly(methylmethacrylate) PMMA cement by Charnley in the 1960s revolutionized orthopaedic medicine. Since this time, however, little has changed. The development of bioactive composites, such as HAPEX^{TM} (a composite of 40% vol hydroxyapatite (HA) in a polyethylene matrix) have potential in orthopaedic applications. The composite has been shown to allow direct bone bonding *in vivo*, and *in vitro* studies have shown preferential attachment to HA exposed on the composite surface. *In vitro* study has also shown that altering the topography HAPEX^{TM} can enhance osteoblast response. This study uses microscopical investigation of osteoblast cytoskeleton, and biochemical measurement of proliferation (by thymidine incorporation) and phenotype (by alkaline phosphatase activity) to compare primary human osteoblast (HOB) activity on HAPEX^{TM} and PMMA cement. The study shows large increases in HOB response to the new generation material compared to PMMA, the current implant standard.

1. Introduction

The advent of poly(methylmethacrylate) (PMMA) bone cement in the 1960s, has revolutionized the success rate of joint replacement arthroplasty in contemporary orthopedics [1, 2]. PMMA cement is a self-curing acrylic polymer that firmly fixes prosthetic components to bone. Despite having a good success rate (90% at 15 years post-implantation), it does have a large number of limitations. These include high exothermic temperature of polymerization (67–124 °C) [3] leading to thermal bone necrosis, and chemical necrosis due to leachable monomers [4]. Furthermore, shrinkage during polymerization also occurs and in addition, it has a much higher modulus compared with adjacent bone leading to interfacial stress formation [5].

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) [6]. PMMA cement is described as brittle, with low material toughness and poor fatigue properties associated with porosity [7].

These problems have lead materials scientists to study bone in greater depth in order to allow the development of next generation, bioactive, materials. Bone can be considered an approximately 50% (vol) hydroxyapatite (HA)-reinforced collagen composite, with HA acting as the major load bearing component, and collagen acting to stop the brittle fracture associated with 100% HA. To develop a bone replacement material, Bonfield *et al*. [8], chose high-density polyethylene (HDPE) with a modulus of ~ 1 GPa as the matrix for an HA filler (Young's modulus of ~ 81 GPa). HA is a phosphate ceramic that resembles bone mineral. It has a reactive surface, producing biocompatibility and bioactivity [9–12]. The composite comprises 40% vol HA in HDPE, and has a Young's modulus of 4.4 ± 0.7 GPa. This approaches the lower end of the modulus values obtained for cortical bone (7–30 GPa). The composite has the trade-name HAPEX^{TM}, and fails in a ductile manner (as opposed to a brittle manner) [1, 13]. HAPEX^{TM} has been shown to support osteoblast growth *in vitro*, and to give direct bone growth *in vivo*. It has also established clinical uses for middle ear and orbital floor implants [13, 14].

Material surface topography is known to be important in cell–material interaction, for cell orientation and migration. Different cell types respond to grooves, pits, pillars and porous topographies [15–19]. Osteoblastic phenotype and degree of bone contact have been shown

*Author to whom all correspondence should be address. Present address: Centre for Cell Engineering, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, UK.
†Present address: Department of Materials Science and Metallurgy, University of Cambridge, Pembroke Street, Cambridge, CB2 3QZ, UK.

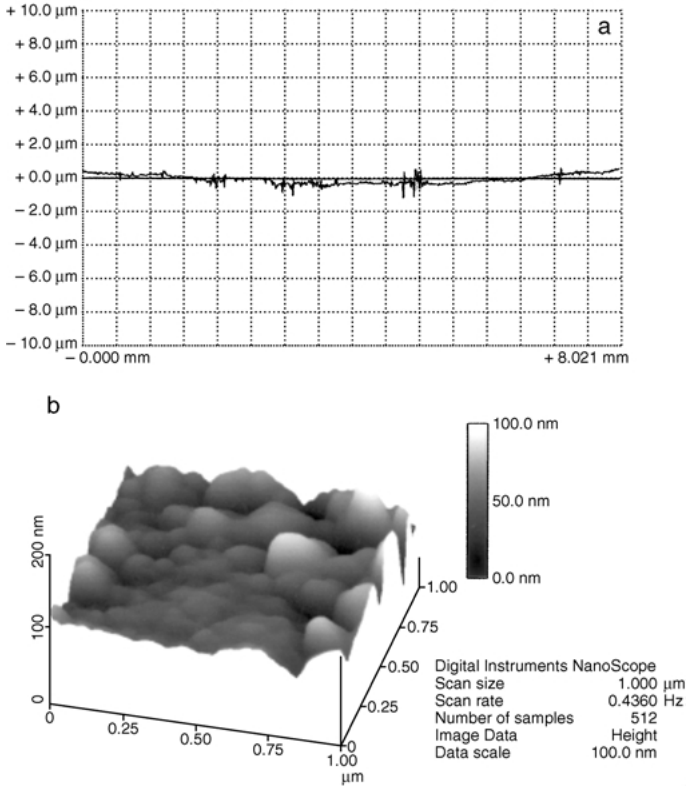

Figure 1 Topographical data for the enhanced HAPEX^{TM} surface. (a) Talysurf trace and (b) AFM image showing that the surface produced by first polishing and then abrading has many naometric features.

to react to topography, with polished surfaces producing lower levels of material–bone contact, and bone formation preferentially observed in the base of grooves and crevices [20].

Previous *in vitro* studies with HAPEX^{TM}, have shown that surface roughness of the composite effects osteoblast response. By first polishing the samples to expose the HA, and then sonicating the surface with alumina to add texture, significantly increased cell attachment, proliferation and differentiation was achieved over a given time period [21, 22].

This study compared second (optimized topography HAPEX^{TM}) and first (PMMA) generation implant materials *in vitro* using primary human osteoblasts (HOB) as a representative cell model. Confocal microscopy has been used to study actin cytoskeleton and vinculin involvement in focal adhesions. Tritiated thymidine incorporation (^{3}H-TdR) and alkaline phosphatase (ALP) have been used to measure proliferation and differentiation on the materials.

2. Materials and methods

2.1. Materials

HAPEX^{TM}: HA/HDPE at 40% vol HA (Plasma Biotal, Tideswell, UK) was produced by incorporation of HA into HDPE (BP Chemicals International, East Riding, UK) through twin screw extrusion (BTS40L: Betol, Luton, UK) and compression molding. The mean HA particle size used was 4 µm, and the particles were relatively acicular, with morphology retained after processing. Disks 12 mm in diameter were machined from the molded blocks. The disks were then polished to 1 µm (polished with graded sandpaper and 1 µm diamond paste on a Struers Abramin (Rodovre, Denmark). After polishing, the disks were roughned in an ultrasonic bath (Engisonic; Engis, Reading, UK) for 4 h with 4 g of polishing grade alumina (4 µm particle size; Sigma, St. Louis, MO) with 4 ml of ultrapure water. The samples were washed in water to remove any residual alumina. A full characterization of the surface has been previously described in Dalby *et al*., 2002 [21]; briefly, the samples were checked over an 8 mm distance with a Talysurf series 2 (FTS4C; Taylor Hobson, Leicester, UK) form and surface analyzer and by Nanoscope IIIa atomic force microscope (AFM) in contact mode (Fig. 1).

PMMA: PMMA cement disks (Coripharm GmbH, Germany), 1.2 cm in diameter, were prepared by addition of MMA monomer to PMMA powder. The mixture was stirred under controlled temperature conditions ($22 \pm 2\,^{\circ}$C) until the mixture became wet enough to spatula into molds, where they set as cast. The PMMA was then heat cured to prevent leaching of non-reactive monomer. The cement surface was left as-cast to represent its surface texture as used clinically. These surfaces have been previously characterized by scanning electron microscopy (SEM) and EDAX [23–27].

Both HAPEX^{TM} and PMMA disks were sterilized by gamma irradiation at a dose of 2.5 Mrad (Swann Morton, UK).

2.2. *In vitro* cell culture

Primary 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% fetal 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 fragments were further chopped with scalpel blades, and incubated in complete medium at 37 °C, 5% CO_2 in a humid atmosphere. Once an osteoid seam of cells transferring from the fragments to the culture plastic was observed, the fragments were transferred to a PBS solution containing collagenase (100 U/ml) and trypsin (300 U/ml) (0.01 M HEPES buffered). The bone fragments were 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 ALP (biochemical and histochemical), osteocalcin, procollagen type I, and response to parathyroid hormone (measurement of cAMP) [28].

For this report, HOB cells were cultured on the test materials and control Thermanox (TMX, Life Technologies) at 2×10^6 cells cm^{-2} for 1, 3, 7, 14, and 28 days for biochemical analysis, and for three days for fluorescence microscopy, under conditions described in a previous study [14].

2.3. Cell growth and differentiation

Cell growth and proliferation were assessed using total DNA and tritiated thymidine (^{3}H-TdR) incorporation, in each case $n = 5$ replicates. HOB phenotype was determined biochemically using a COBAS-BIO (Roche, UK) centrifugal analyzer for ALP measurement. These methods have been described in a previous study [14].

2.4. Immunofluorescence of vinculin and actin

After three days of culture, the cells on the test materials 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 min 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 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

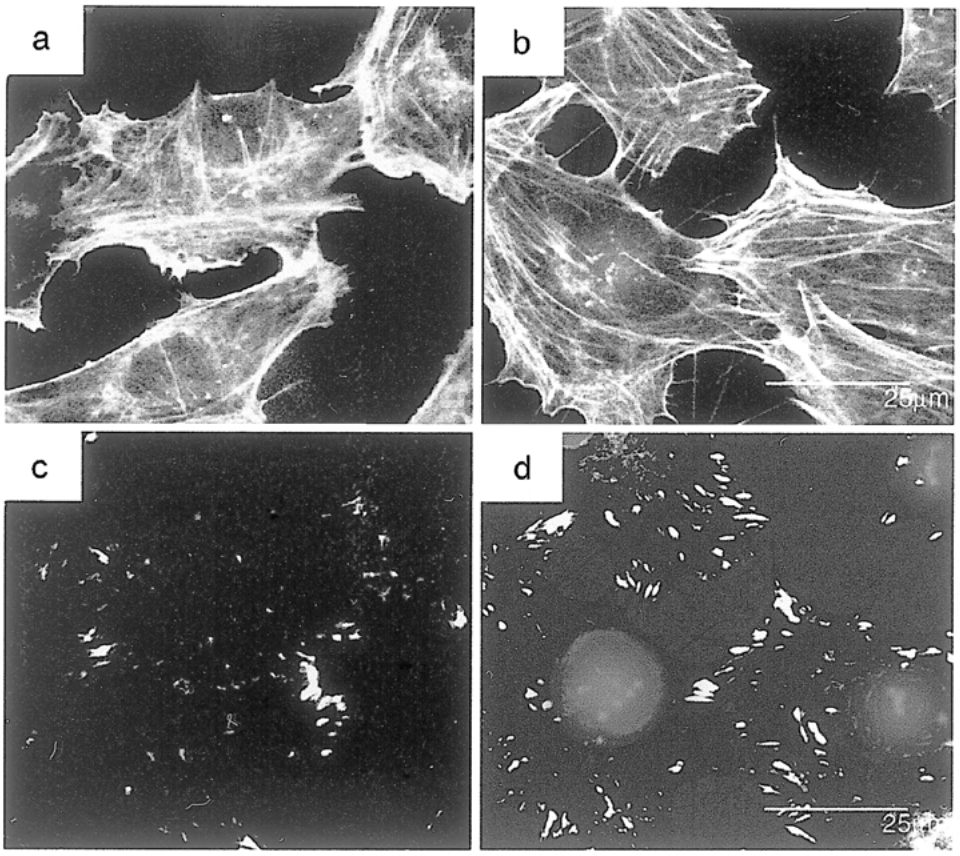

Figure 2 CLSM images for HOBs cultured on enhanced topography HAPEX™ and PMMA. Cells cultured on PMMA had a diffuse actin cytoskeleton (a), and few focal contacts (c). Cells cultured on HAPEX™ had a well defined actin cytoskeleton with many stress fibres (b), and many more focal contacts (d).

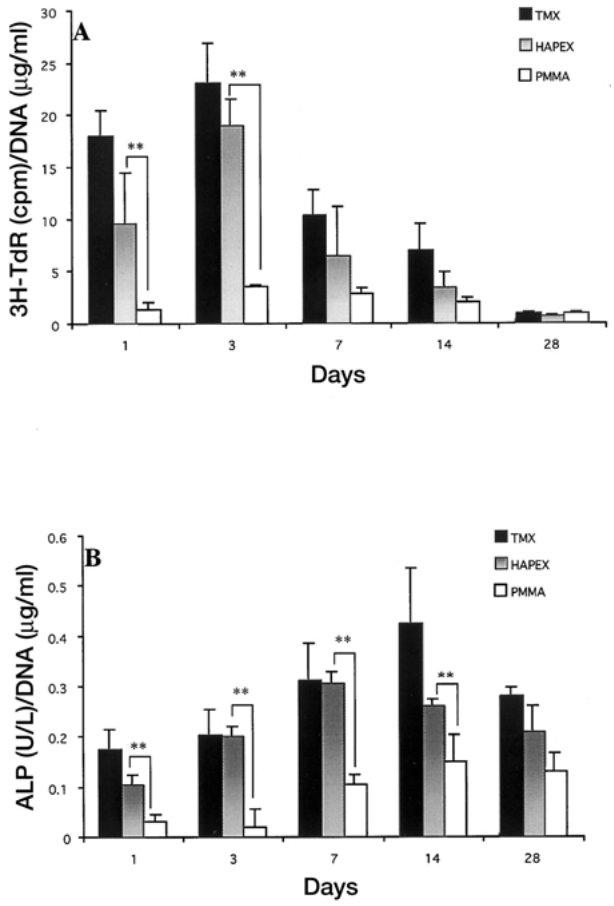

Figure 3 Graphs for HOB proliferation (a) and differentiation (b) in response to enhanced topography HAPEX^{TM} and PMMA cement. Increased levels of ^{3}H-TdR uptake and ALP activity were seen on HAPEX^{TM} compared to PMMA. $n = 5$, ** $= p < 0.01$.

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. Statistics

A multiple comparison statistics test, Tukey Kramer honestly significant difference, was used for statistical analysis using SPSS (a Windows-based software). This is a one way ANOVA for non-parametric data. Note: For clarity, the statistics for TMX are not shown in the figures, but are mentioned in the results text.

3. Results

The results for actin cytoskeleton and vinculin in focal contacts showed large differences in the quality of cell adhesion on PMMA and HAPEX^{TM}. The results for HAPEX^{TM} showed many focal contacts in response to the material (Fig. 2(d)), the actin cytoskeleton was well organized with many stress fibers seen throughout the cells (Fig. 2(b)), The results for PMMA, however, showed very few focal contacts being expressed in the cells (Fig. 2(c)), and the microfilaments were less organized and more diffuse (Fig. 2(d)).

^{3}H-TdR incorporation studies showed HAPEX^{TM} to support significantly higher HOB proliferation than PMMA at days 1 and 3 (Fig. 3(a)). The cells cultured on HAPEX^{TM} followed the same trend as those cultured on TMX. A significant difference between cells cultured on TMX and HAPEX^{TM} was observed only on day 1, whereas HOBs on TMX were significantly more proliferative than those on PMMA on days 1, 3, 7 and 14. All samples showed a highest level of HOB proliferation on day 3 (Fig. 3(a)).

ALP activity, however, was seen to be highest for HAPEX^{TM} on day 7 and for TMX and PMMA on day 14, gradually increasing from day 1, then falling again by day 28 of culture. ALP activities observed on TMX were significantly greater than those observed on HAPEX^{TM} at days 1 and 14, and were greater than for cells on PMMA at all time points. HOB ALP activities were significantly greater than those obtained for osteoblasts on PMMA on days 1, 3, 7 and 14 (Fig. 3(b)).

4. Discussion

When a material is implanted *in vivo*, or placed into culture medium *in vitro*, it immediately reacts with serum proteins, and any cellular event that follows is due to the type and nature of protein adsorbed onto the material surface [28]. The surface chemistry of the material will determine the subsequent protein/material interactions, and thus the incorporation of bioactive components, such as HA, can facilitate increased cellular response. PMMA and HDPE have both been described as inert, but it is to be noted that no material can truly be inert [28]. The term inert suggests that the material will not react with extracellular matrix proteins, and thus will not allow cell adhesion, in practice however, some cells are recruited and low levels of proliferation and differentiation are seen (as for PMMA in the results section).

Addition of HA is especially beneficial for orthopaedic applications as it has a similar chemistry to that of native bone apatite, and thus stimulates osteoblast adhesion [9–12]. Previous studies with HAPEX^{TM} have shown that HOBs produce filopodia in response to the composite, and that these microspikes preferentially adhere to exposed HA. This suggests that the HA is recruiting proteins that facilitate HOB adhesion, in preference to the inert HDPE [13, 20, 21, 29].

In accordance to the above statement, HOB adhesion, as shown by vinculin labeling, was improved for cells on HAPEX^{TM} compared to PMMA. Vinculin acts as a linker protein, in the assembly of the focal contacts, between transmembrane integrin proteins and the actin cytoskeleton [30]. Integrins are the linker proteins between the cell and the material, and are involved in cell–material signaling events [30]. The cells are stimulated by integrin–absorbed protein binding, causing formation of F-actin stress fibers. Polymerization of actin in to stress fibers, and their subsequent contractile action, gathers the integrins, hence forming the focal contacts observed. The number of adhesion plaques formed, directly effect subsequent cell response through actin cytoskeleton, and G-protein signaling cascades, initiated at the contacts

altering transcription events within the nucleus, and hence protein production from the cells [30–32].

Topographical studies using gene microarrays, have shown activation of G-protein signaling and increases in transcription factor levels in response to surfaces that increase cell proliferation [16]. In this study, increased expression of focal contacts has indeed resulted in increased proliferation on HAPEX^{TM} compared to PMMA. The initial recruitment and proliferation of cells onto an implant surface is of great importance for the long-term material success [33, 34].

The HOBs cultured on HAPEX^{TM} have also significantly greater ALP activities than those cultured on PMMA. ALP is expressed by osteoblasts during bone formation. It is thus a good indicator of bone formation activity. The enzyme has roles in elevating calcium and phosphate levels to the point of spontaneous precipitation [35].

The similarities in proliferation and differentiation trends observed between HAPEX^{TM} and TMX suggests that HAPEX^{TM} is supporting high levels of cell growth, as TMX is a control for negative cytotoxicity. It is seen from Fig. 3, however, that HOB activity on PMMA lags far behind that of topography enhanced HAPEX^{TM}.

5. Conclusions

The results clearly show the biological improvement that is becoming possible with the development of new biomedical materials, such as HAPEX^{TM}. Medicine needs to progress from the use of inert materials towards the use of next generation materials that have been designed to elicit appropriate responses in specific applications. By exploiting available methods for increasing biological activities, it is hoped that the effective life span of implants will, in the near future, be significantly improved.

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