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Osteoblast response to polymethyl methacrylate bioactive glass composite

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Abstract Polymethylmethacrylate (PMMA) has been used in many orthopedic and dental applications since the 1960s. Biocompatibility of newly developed surface porous fiber reinforced (SPFR) PMMA based composite has not been previously proven in cell culture environment. Analysis of rat bone marrow stromal cells grown on the different test materials showed only little difference in normalized cell activity or bone sialoprotein (BSP) production between the test materials, but the osteocalcin (OC) levels remained higher ($P < 0.015$–0.005) through out the test with SPFR-material when compared to tissue culture poly styrene (TCPS). The cells grown on SP-FRC material also showed highest calcium depletion from the culture medium ($P < 0.026$–0.001) when compared to all other test substrates. SEM images of the cultured samples confirmed that all the materials enabled cell spreading and growth on their surface, but the roughened surface remarkably enhanced this process of cell attachment, division and calcified nodule formation. This study shows that the SP-FRC composite material does not elicit harmful/toxic reactions in cell cultures more than neutral TCPS and can be considered biocompatible. The material possesses good capabilities to form new mineralized tissue onto its surface, and through that a possibility to bond directly to bone. Rough surface seems to enhance osteoblast proliferation and formation of mineralized extracellular matrix.

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1 Introduction

Bone loss in orthopedic and trauma surgery possesses a challenge for traditional surgical bone replacement with autogenous and allogenous bone grafts. Alternative synthetic materials for bone repair have already been presented in cranio-maxillo-facial, spinal and experimental surgery with promising results [1–5]. Bone cements based on polymethyl methacrylate (PMMA) have been used in orthopedics to secure implants into the skeleton and fill bone voids after tumors or trauma since the 1960s, and they still remain as the method of choice today [6–8]. Certain improvements to the bone cements have been suggested by including porogens and reinforcing fillers to the cement [9, 10].

In orthopedic operations the PMMA cement is autopolymerized in situ—i.e. the mixed cement dough of PMMA powder and methyl methacrylate (MMA) monomer with initiators and activators is placed in the body and left to polymerize. During exothermal polymerization process excess heat up to +70°C, or even higher temperatures, is generated and dissipated into the surrounding tissues [11].

Tissue damage can occur already between +42 and +47°C trough coagulation of the proteins [12]. PMMA has been under a lot of investigations since the 1970s because of the adverse cardiovascular and thromboembolic effects by the monomers of MMA that the in vivo/in situ

polymerization can cause during and shortly after operations [13, 14]. The leaching of free monomers before polymerization, leaching of residual monomers after polymerization, leaching of residual of initiators and activators have been associated with inhibition of cell function and growth/differentiation, increased release of inflammatory cytokines, and cell death/necrosis which can lead to the loosening of the prosthesis/implants [15–17]. The amount of released monomers is related to the polymerization method—i.e. in vivo/in situ curing, pressure curing or heat polymerization. It is known that heat induced polymerization of PMMA produces polymer with higher degree of monomer conversion and lower quantity of residuals [18–20].

In dental and orthopedic applications fiber reinforcements have been successfully combined with PMMA to enhance the polymer's mechanical properties [9, 21–25]. Fiber reinforcement or addition of bioactive filler lowers the setting/polymerizing temperatures by reducing the quantity of polymerizable monomer resin system, although the release of residual monomers has been shown to increase slightly by incorporation of additional fillers [26, 27]. The majority of the remaining residual MMA in PMMA based cements has been shown to wash out during the first 24–72 h of storage in water (37°C) [28, 29].

The favorable effects of surface roughness and porosity for bone on growth, and osteoblast attachment and function have been proven in earlier studies. This biological effect goes beyond the mere mechanical locking provided by bony ingrowth into porosities, by enhancing osteoblast adhesion, activation, migration, and proliferation as well as matrix and mineral secretion [30–32].

The surface porous fiber reinforced (SPFR) implant has been developed for segmental long bone defects in load bearing areas. It is made of PMMA core with E-glass fiber-reinforcement, and bioactive glass (BAG) particles on the surface [1]. The current study was done to evaluate the biocompatibility of a combination of certain significant components of the SPFR -material. The effects of the rough surface topography and addition of BAG filler were evaluated in rat bone marrow derived osteoblast cultures. The cell proliferation, functions and extracellular matrix production were evaluated by soluble ingredients and scanning electron microscope (SEM). For the cell cultures three different types of disks were fabricated: (1) smooth PMMA (S-PMMA), (2) rough surface PMMA (R-PMMA) and (3) rough surface PMMA with BAG (BAG-PMMA) as a simplified combination of the of the components to simulate SPFR-material.

Tissue culture poly styrene (TCPS) disks were used as control materials. The disks were pre-polymerized and washed extensively before use, both to minimize the quantity of MMA and activator/initiator residuals.

2 Materials and methods

Neat PMMA specimens and composites with bioactive glass granules were prepared as follows. PMMA powder (Palapress©, Heraeus Kulzer GmbH & Co KG, Hanau, Germany) containing benzoyl peroxide initiators and MMA monomer (Fluka Chemie GmbH, Buchs, Switzerland) containing 2 wt% *N*,*N*-dimethyl-*p*-toluidin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) as activator were mixed together (liquid-to-powder ratio 1:1 by weight). For PMMA-bioactive glass (BAG) specimens (Group: PMMA-BAG), 30 wt% of (BAG) granules (S53P4, size: 315–500 μm, Vivoxid Ltd. Turku, Finland) were added to the mixture. The PMMA-MMA or PMMA-MMA-BAG mixture was poured into a disposable syringe to fabricate cylindrical specimens and the mixture was polymerised in a pressure-curing device (Ivomat, Typ IP 2, Ivoclar AG., Schaan, Liechtenstein) at a pressure of 400 kPa, at a temperature of 90 ± 3°C, for 20 min. The polymerized specimens (diameter: 12 mm) were taken out of the syringe and sawed into disks (height: 3 mm). Half of the polymer disks were roughened (Group: R-PMMA) with 180 grit silicon carbide grinding paper, and the other half [smooth PMMA group (Group: S-PMMA)] were polished with 2,400 grit using a grinding machine (LaboPol-21, Struers A/S, Rødovre, Denmark). All BAG-PMMA composite disks were roughened. After ultrasonic cleaning with 70% ethanol for 5 min the specimens were washed with distilled water and sterilized in an autoclave at 120°C for 20 min. Conventional tissue culture polystyrene (TCPS; Costar, Corning Inc, NY) wells were used as controls.

2.1 Cell cultures

Rat bone marrow stromal cells were harvested and cultured according to Maniatopoulos et al. [33]. Briefly, the femurs of three young male adult Sprague–Dawley rats were isolated. The bones were wiped with 70% alcohol and immersed twice in α-MEM (Sigma Chemical Co., USA) culture medium containing 100 units/ml of penicillin/streptomycin (Gibco BRL, Life Technologies BV, The Netherlands). The condyles were cut off and bone marrow was flushed out using complete cell culture medium (α-MEM and antibiotics supplemented with 15% fetal bovine serum (Gibco), 50 μg/ml ascorbic acid (Sigma), 5 mM Na-β-glycerophosphate (Merck, Germany), and 10 nM dexamethasone (Sigma)). The resulting suspension was passed through a 22 gauge needle, and plated cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

After 7 days of primary culture, the adherent cell population was trypsinized and resuspended in complete culture medium. Polymer and composite substrates were washed once with phosphate buffered saline (PBS) and

once with culture medium at 37°C, for 1 h each. Cell suspension was subsequently added on the test substrates at a density of 25,000 cells/cm^2, and cells were allowed to adhere for over night. After seeding, osteoblast culture was continued for 3 weeks in 24-well plates with medium replacement every 2–3 days.

2.2 Cell activity

Proliferation of cultured cells was determined using AlamarBlue™ assay (BioSource International, USA) in colorimetric format. Specimens (n = 4) were withdrawn from the culture at predetermined time-points, washed in PBS, and placed to clean 24-wells. Fresh culture medium with 10% assay reagent was added to the wells, and after 3 h incubation, absorbance values of the medium were read at 560 and 595 nm using an ELISA plate reader (Multiskan MS, Labsystems, Finland). Measured absorbances were used to calculate the reduction of assay reagent, and the cell activities were normalized in respect to those on TCPS at the first time-point—day 1 represents 100% activity. Reductive cell activity of cultured osteoblasts has been shown to correlate with their numbers [34].

2.3 Mineralization

Calcium concentrations in used culture media were measured to follow the osteoblast mineralization. Calcium concentrations were determined using ortho-cresolphthalein complex one (OCPC) method [35]. The assay reagent consisted of OCPC with 8-hydroxyquinol in an ethanolamine/boric acid buffer. Absorbances were taken at 560 nm using the ELISA plate reader and calcium concentrations were obtained from a $CaCl_2$ standard curve.

2.4 RT–PCR

At predetermined time-points, total cellular RNA from TCPS control wells and polyA mRNA from the experimental culture substrates were isolated using Trizol® reagent (Gibco) and QuickPick™ mRNA magnetic beads (Bio-Nobile, Finland), respectively. Three replicate RNA pools from each substrate type were reverse transcribed with random hexamer primers using GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems, USA). The resultant first-strand cDNA was analyzed in duplicate PCR reactions using iQ Supermix kit (Bio-Rad Laboratories) and FAM-labeled TaqMan® Gene Expression Assays (Applied Biosystems) for bone sialoprotein (BSP; Rn00561414_m1), osteocalcin (OC; Rn00566386_g1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a control gene; Rn99999916_s1). PCRs were carried out using an iCycler iQ real-time PCR detection system with software version 3.1 (Bio-Rad Laboratories). The following cycling conditions were used: 95°C/5 min; 40 cycles of 95°C/20 s, 60°C/60 s. The threshold cycles (C_T) were automatically calculated using "the maximum curvature approach" and gene expression levels of BSP and OC were normalized to GAPDH expression in each RNA pool ($\Delta C_T = C_{T,\ target} - C_{T,\ GAPDH}$). A difference of one unit in ΔC_T values corresponds to a two fold difference in gene expression level.

2.5 Scanning electron microscopy

Scanning electron microscopy (SEM; JSM-5500, Jeol Ltd, Japan) was used to study the progression of osteoblast cultures. The examination was done after 1, 4, 7 and 14 days of incubation, using samples of each specimen type/time-point. Cell culture specimens were washed in PBS and fixed with 2% glutardialdehyde in a 100 mM cacodylic acid buffer pH 7.4. The fixed substrates were dried in a rising alcohol series, stored in an exicator and sputter coated with carbon (BalTec CED 030, BalTec Limited, Balzers, Liechtenstein) for imaging. The SEM imaging was done using an accelerating voltage of ~12–14 keV.

2.6 Statistics

Statistical analyses were performed using the SPSS v.16.0 software package for MAC (SPSS Inc., Chicago, IL, USA). The data was analyzed with one-way ANOVA followed by Tukey's post-hoc test. Differences were considered significant at 95% confidence level.

3 Results

3.1 Proliferation and mineralization

SEM images (Fig. 1) from the cultured populations at day one did not reveal any difference between the material groups, as only few clusters of cells could be seen attached onto the specimen surface. By the 4th day the R-PMMA and BAG-PMMA groups exhibited cell proliferation, and the cells spread over the surface and began to fill the grooves on the materials. S-PMMA group exhibited only minor cell proliferation, and still had moving cells on the surface (pseudopodia). By day 7, cell proliferation on R-PMMA and BAG-PMMA had covered the materials grooves, and began to show calcified nodule formation. S-PMMA surfaces were covered with single cell lining, with large empty areas and moving cells Observations at day 14 revealed that a thick cell lining with good formation of calcification centers covering all the materials.

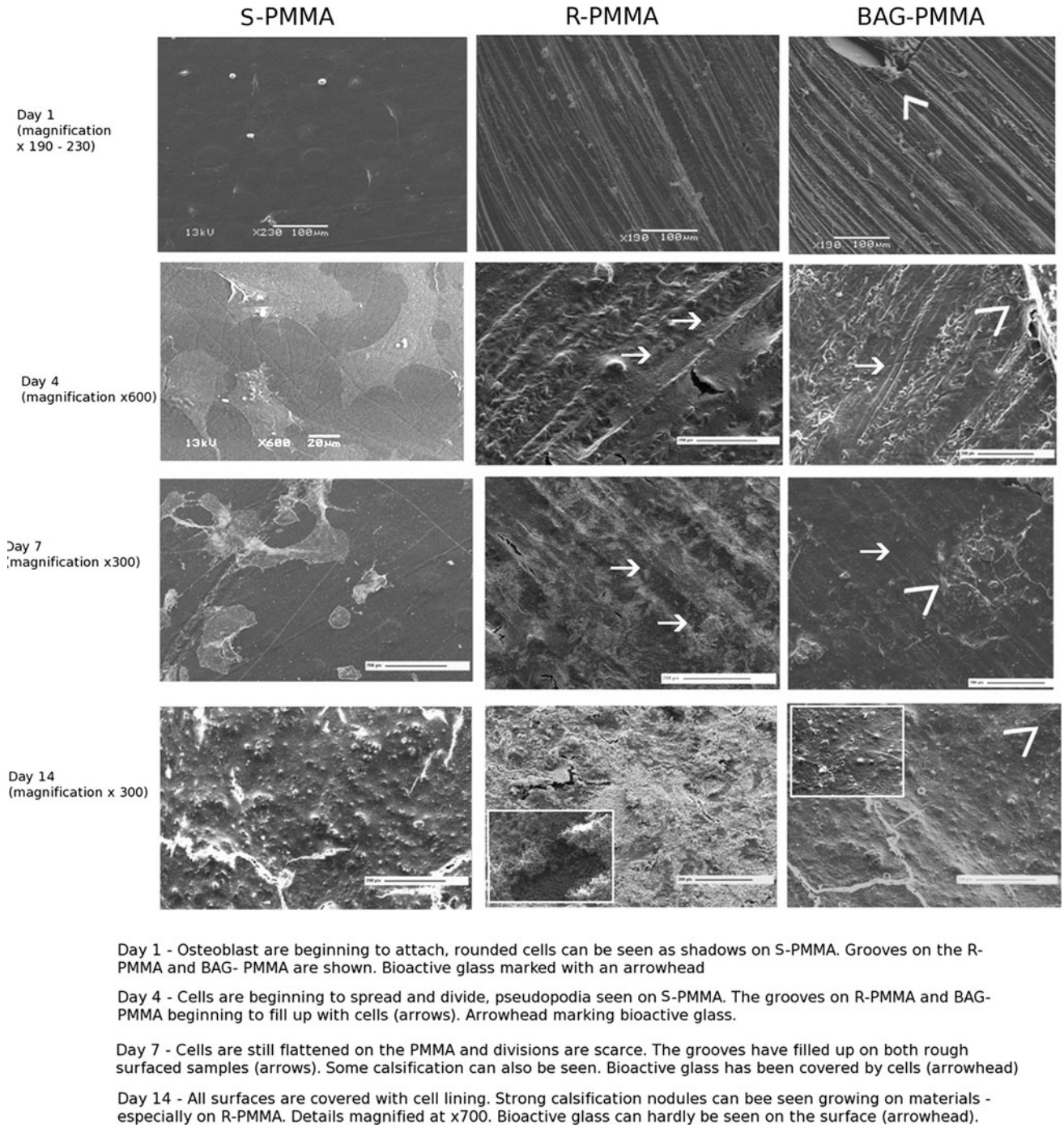

Fig. 1 SEM images of cell proliferation and extracellular matrix production on culture disks at determined time-points

The R-PMMA material showed the most abundant formation of calcium deposits, which grew high up from the material surface (Fig. 1).

Cell activities on S-PMMA showed statistically significant increase for only 4 days, whereas cell activities on the other substrates increased up to 7 days (Fig. 2). By day 7 the cell activity on R-PMMA was significantly greater than on S-PMMA or TCPS substrate. At later times the cell activities remained unchanged or began to decline. Greatest decline in cell activity by day 21 was observed in R-PMMA group, where it was reduced significantly when compared to S-PMMA or BAG-PMMA groups ($P = 0.027$ and $P = 0.041$ respectively).

Evolution of calcium concentrations in cell culture medium is summarized in Fig. 3. Soluble ions were released from the BAG-PMMA resulting significantly

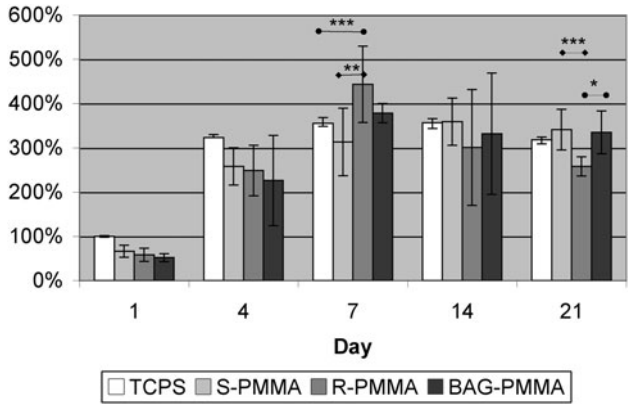

Fig. 2 Cell activities in the osteoblast culture. The reduction of AlamarBlue™ reagent with TCPS at 1 day time-point was set to 100%. The *bars* represent standard deviations and statistically significant differences between time-points are indicated above the columns (* $P < 0.05$, ** $P < 0.04$, *** $P < 0.03$)

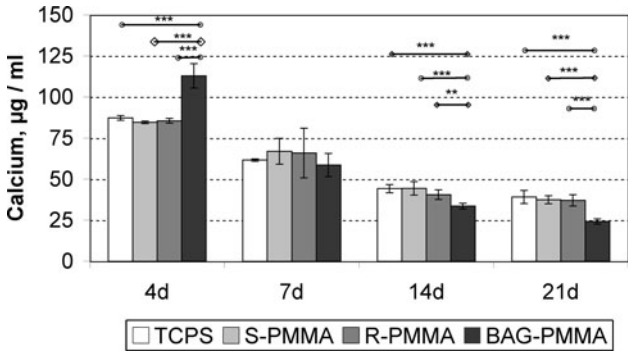

Fig. 3 Calcium concentration in cell culture medium in mineralizing osteoblast cultures. The calcium concentration of fresh culture medium is ~85 µg/ml. The *bars* represent standard deviations. Statistically significant differences in changes of the calcium concentrations between culture mediums at different time-points are indicated above the columns (** $P < 0.03$, *** $P < 0.001$)

($P < 0.001$) increased calcium concentrations compared to all other mediums at day 4. Within 7 days, osteoblasts on all substrate types had started to mineralize (Fig. 1), which was also noted as depletion of calcium from the culture medium. Significantly lowest soluble calcium concentrations in cultures—suggesting the strongest mineralization/uptake of Ca-ions—were observed in BAG-PMMA group ($P < 0.001$) when compared to all of the other substrates both at day 14 and 21.

3.2 Gene expression

The cell stock used to seed the substrates exhibited high BSP expression ($\Delta C_T = 8.1$) but low OC expression ($\Delta C_T = 0.2$). After 1 week of culture, the BSP expression level was slightly declined with all substrate types (ΔC_T ranged from 5.2 ± 0.4 to 6.2 ± 0.5), with no further

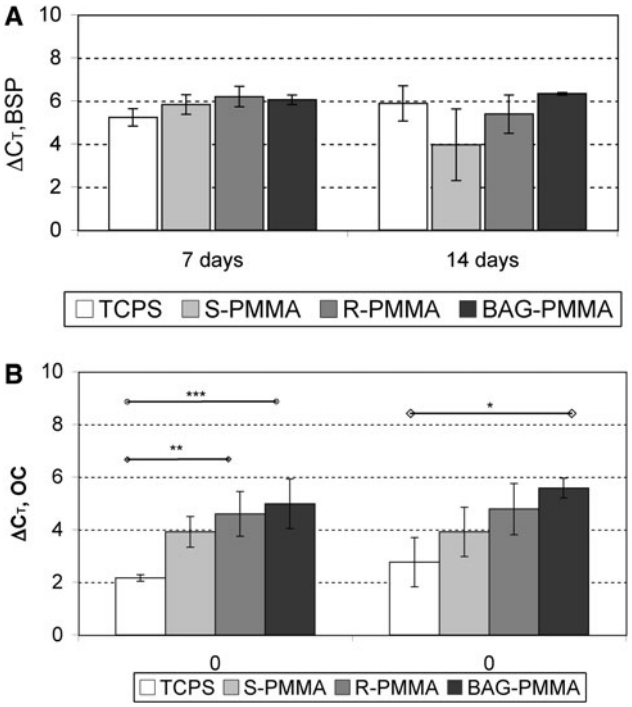

Fig. 4 Bone sialoprotein (**a**) and osteocalcin (**b**) gene expression. The *bars* represent standard deviations and statistically significant differences between substrate types are indicated above the columns (*** $P = 0.005$, ** $P = 0.012$, * $P = 0.015$)

change trough out the latter part of the test (7–14 days). No significant difference in BSP expression could be found between the substrate types at any time (Fig. 4a). The OC expression was induced (ΔC_T ranged from 2.2 ± 0.1 to 5.0 ± 0.9) during the 1st week of culture (Fig. 4b) and remained relatively similar through out the study. At day 7 the expression on (ΔC_T) OC was significantly higher for the BAG-PMMA and R-PMMA groups ($P = 0.005$ and $P = 0.012$ respectively) when compared to TCPS. The highest OC expression was recorded at day 14 with BAG-PMMA substrate, with significant difference to TCPS group ($P = 0.015$). The absence of statistically significant differences between S-PMMA, R-PMMA and BAG-PMMA materials in both BSP and OC expression tests may be due to large standard deviations within the groups, but non the less, the highest expressions were observed in BAG-PMMA group at day 14.

4 Discussion

Many undesired properties have been linked to the PMMA based materials. Direct cytotoxic effects of the free and residual monomers and initiators with activators which are released during the polymerization process and after implantation have been studied in vitro [17, 36]. Also

cardiorespiratory problems during implantation of orthopedic devices with PMMA cement have been reported [37, 38]. The polymerized PMMA wear debris alone (small particles) does not cause cytotoxic effects, but only local inflammatory reaction—dependent on the concentration and particle size—when the particles are digested by the leucocytes [16].

PMMA based polymers with bioactive filler (hydroxyapatite (HA), tricalcium phosphate (TCP), etc.) materials have been tested in vitro and in vivo in the past, and found to be biocompatible and enhance bone growth to some extent. This can be explained through calcium and phosphate ion release and precipitation from the material, which promotes cell activity and attachment to the surface, and the formation of calcification centers (nodules) [39–41]. Glass ionomer cements, glass polyakenoates (GICs) were created to improve bone cement biocompatibility through release of favorable ions, but some of the components used to increase their mechanical strength have been proven to elicit cytotoxic effects [42]. GIC cements have been used in some dental applications, but their mechanical properties aren't sufficient for weight bearing orthopedic applications [12]. Bioactive glass has been proven to possess improved osteoconductive properties and to increase the structural strength when applied to PMMA [1, 43, 44].

To improve mechanical properties of polymer based materials to withstand loads by human body, reinforcing fibers have been added to the polymer [21]. The previous study [45] by the authors focused on this approach and presently it was aimed to analyse biocompatibility of the simplified combination of major components of the surface porous fiber reinforced (SPFR) composite, namely polymer matrix and the bioactive modifier of bioactive glass. Due to considerably different form of reinforcing fibers compared to that of particulate fillers of bioactive glass, the biocompatibility of the fibers will be performed in a separate study.

This study showed that the pre-cured, surface modified fiber reinforced composite (represented by BAG-PMMA) supports the cell functions and growth of isolated osteoblast cells. It does not elicit cytotoxic reactions in cells according to the normalized cell activity and BSP levels when compared to TCPS. Cellular functions were even enhanced when tested for OC and BSP secretion, which have been related to good biocompatibility through normal maturation and function of osteoblasts [46, 47]. Concentration of Ca-ions in culture medium was significantly lower for the BAG-PMMA group than any other group at day 21, which can be interpreted as good uptake and deposition by differentiated, viable and active cells [48].

The observations made with SEM comply with the biochemical tests. At day 7 the cells were mature and cell division had ceased, which can be seen from TCPS culture, and cell activity reached its' peak value. R-PMMA and BAG-PMMA groups showed higher biochemical activity levels, which were confirmed in SEM evaluation as speed of attachment, growth and extracellular matrix production. Cooper et al. [46] have shown normal osteoblast mineralization to occur within 14 days of culture, as was also shown in the SEM evaluation. Nodule formation i.e. calcium deposition seen in cell cultures indicates good biocompatibility of the material [49]. No difference could be made with SEM study between R-PMMA and BAG-PMMA groups at day 14, although BAG-PMMA group had higher OC and BSP levels trough out the test period. Both groups exhibited marked mineralization growing up from the surface (Fig. 1). S-PMMA group showed inferior results according to SEM studies or culture medium derived agents, which could be due to weaker adhesion of cells on smooth surfaces.

Biomechanically, glass fiber reinforced composites—such as SPFR composite material—provide high strength material with bone like flexural modulus [50, 51]. This, along the possibility to change the reinforcing fiber directions, allows the use of fiber-reinforced composites as biomimetic material in repairs of large bone defects. Additional benefits of polymer based composites over metals and ceramics are in possibilities to load bioactive substances to the polymer matrix to be released locally by time [52]. This approach, however, requires further research.

5 Conclusions

Bioactive glass and surface roughness promoted cell proliferation and adhesion on surface modified PMMA-composites, and supported normal extracellular matrix production.

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