# Development of Magnetic Particle Techniques for Long-Term Culture of Bone Cells With Intermittent Mechanical Activation

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***Abstract*—Magnetic particles were coated with RGD and adhered to primary human osteoblasts. During a 21-day culture, the osteoblasts plus adhered magnetic particles underwent a daily exposure to a time-varying magnetic field via a permanent NdFeB magnet, thus applying a direct mechanical stress to the cells ($B_{\max} \sim 60$ mT). After 21 days, preliminary results show that the cells plus magnetic particles were viable and had proliferated. A von-kossa stain showed mineralized bone matrix produced at 21 days in the experimental group whereas the control groups showed no mineralized matrix production. Real-time reverse transcription-polymerase chain reaction at 21 days showed an upregulation of osteopontin from the experimental group in comparison to the control group of cells with adhered particles and no magnet applied. These preliminary results indicate that adherence of RGD-coated 4.5 $\mu$m ferromagnetic particles to primary human osteoblasts does not initiate cell necrosis up to 21 days *in vitro*. Also, mechanical stimulation of human osteoblasts by magnetic particle technology appears to have an influence on osteoblastic activity.**

***Index Terms*—Bone, magnetic microparticles, mechanotransduction, osteoblast.**

## I. Introduction

MECHANICAL forces applied to osteoblasts by fluid flow, axial compression, and tension have been shown to induce matrix production, upregulate bone-related gene production, and increase osteo-related protein production [1]–[5].

For tissue engineering purposes, it may be beneficial to apply mechanical forces to osteoblasts seeded onto three-dimensional scaffolds to 1) upregulate matrix production to shorten the time needed in the laboratory to prepare the tissue engineered construct prior to patient implantation and 2) mechanically precondition the osteoblasts prior to implantation into a weight bearing situation. Mechanical loading via cylical, axial compression of tissue-engineered constructs have been performed previously for these reasons [6]. There are, however, limitations as to the type of biocompatible scaffold suitable for this application due to the scaffolds' mechanical properties. A suitable scaffold must be able to withstand the loads applied during *ex vivo* mechanical preconditioning. However, if the mechanical loads can be applied via cell membrane stretching using magnetic microparticle and nanoparticle technology then the limitation of the mechanical properties of the biocompatible scaffold is significantly reduced.

It is possible to apply mechanical strains directly to the cell using magnetic particles. Magnetic microparticles and nanoparticles have been used in medicine for a variety of applications. Cell and cell-product isolation techniques have made use of this technology by binding specific proteins to the particles to target particular receptors [7], [8]. Diagnostics also have utilized this approach using magnetic particles as a contrast medium in radiological imaging techniques such as magnetic resonance imaging [9], [10] and such particles also have been used as a drug delivery agent [11].

In order to apply a mechanical stress, the magnetic particles are coated with a protein to allow adherence to the cell membrane and a time-varying magnetic field is applied. In the case of magnetically blocked particles, the application of a magnetic field at an angle to the particle's magnetization vector will apply a torque $(\tau)$ to the particle according to

$$\tau = \mu \mathbf{B} \sin\theta \tag{1}$$

where $\tau$ is the torque on the particle, $\mu$ is the magnetic moment of the particle, $\mathbf{B}$ is the magnetic flux density, and $\theta$ is the angle between the particle's magnetization vector and the field vector. The cell's membrane will deform in response to the torque applied to particles which attached to it [12].

In addition to the torque applied to blocked particles (the blocking volume depends on the material), all magnetic particles (including superparamagnetic nanoparticles) will experience a translational force $(F_{\mathrm{mag}})$ in the presence of a gradient field according to

$$F_{\mathrm{mag}} = (\chi_2 - \chi_1) V \frac{1}{\mu_o} B(\nabla B) \tag{2}$$

where here $\chi_2$ is the volume magnetic susceptibility of the magnetic particle attached to the cell, $\chi_1$ is the volume magnetic susceptibility of the surrounding medium (i.e., tissue/bone), $\mu_o$ is the magnetic permeability of free space, and $\mathbf{B}$ is the magnetic flux density in Teslas (T). Though this assumes spherical particles and no magnetic dipole interactions, it should give a good approximation of the field and gradient required for the system.

These forces (translational and/or rotational) apply a deformational stress directly to the cell membrane. Application of

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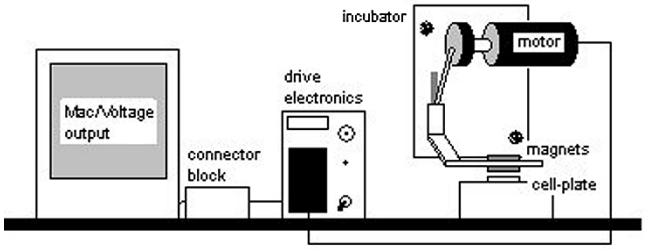


Fig. 1. Schematic of setup used to apply cyclical permanent magnet magnetic field to cells with adhered magnetic particles in six-well plate.

mechanical stress to cells has been performed in this way using magnetic particles that have been coated in a variety of proteins such as collagen type1 [13], RGD [14], fibronectin [15], bovine serum albumin [16], and poly-l-lysine [17]. Translational stretches and torques have been applied to individual cells in this manner and an upregulation of $Ca^{2+}$ influx into cells [15], [16] and significant alterations in the cytoskeletal network such as actin filament stiffening [16], [18] has been reported as a result of such strains.

Mechanical stress application to cells using magnetic particle technology has not been studied in the long term. Also, this technique has only been applied to single cells. In this paper, we have used this mechanotransduction method on a multiple cell population rather than just single cells. We have studied the effects of applying a mechanical stress directly to primary human osteoblasts for a 21-day period using this approach.

## II. Methodology

Ferromagnetic $(CrO_2)$ particles ($\text{diameter} = 4$–$4.5\ \mu$m, Spherotech) were coated with RGD (50 $\mu$g/ml PBS) and adhered to primary human osteoblasts at a concentration of approximately two particles per cell. The particles were added while the cells were in suspension and were then plated at a number of approximately 100 000 cells (plus adhered particles) into wells of a six-well plate. These cells were then grown in the presence of culture medium (alpha-MEM, GIBCO), 1% antibiotic-antimycotic solution (GIBCO), 10% fetal calf serum and osteogenic supplements of $10^{-8}$ M Dexamethasone (SIGMA), 10 mM $\beta$-glycerophosphate (SIGMA), and 50 $\mu$g/ml ascorbic acid (SIGMA) for 21 days. Culture medium was changed every two days. Six sample groups were analyzed as described in Table I. A 1-Hz/60 mT, (max.) magnetic field generated by an oscillating NdFeB magnet array was applied to the cells plus adhered particles each day for 30 min using a computer-controlled drive system as described in Fig. 1. The drive system was at room temperature and so the samples were removed from the incubator in order for the magnetic field to be applied. Control plates to allow for this change in environment for 30 min daily were included in the experiment (group NoMNoP as seen in Table I).

At 21 days, live/dead fluorescent staining (propridium iodide/syto9, Molecular Probes) and scanning electron microscopy (SEM) analysis were performed to evaluate cell viability and morphology. At a two-day and one-week time point, cells with approximately two particles per cell were analyzed with Laser scanning confocal microscopy (LSCM). Actin filaments were stained with FITC labeled phalloidin (SIGMA) after permeabilization using 0.05% TritonX100 (BDH) in phosphate buffered saline (SIGMA). The nuclei of the cells were stained blue using Vectashield Mounting Medium with DAPI (Vectorlabs). Light microscopy also was performed to show the position of the beads on the cells.

TABLE I
Description of Six Sample Groups

| Sample Group Number | Sample Group Abbreviation | Sample Group Description |
|---|---|---|
| (1) | ALL | Cells plus particles, with applied magnetic field, with 30 minutes at room temperature (as magnet was applied at room temperature for 30 minutes). |
| (2) | NoP | Cells with no particles, with applied magnetic field, with 30 minutes at room temperature. |
| (3) | NoM | Cells with particles, with 30 minutes at room temperature, with no magnetic field applied. |
| (4) | NoMNoP | Cells with no particles, with 30 minutes at room temperature, with no magnetic field applied. |
| (5) | CellsOnly | Cells with no particles, with no 30 minutes at room temperature, with no magnetic field applied. |
| (6) | NoCells | No Cells, no magnetic field, no 30 minutes room temperature. |

A Prostaglandin $E_2$ ELISA (Metachem Diagnostics Ltd.) was performed on the three-day supernatant retrieved from four samples of each of the six groups analyzed as described in Table I. This assay was performed as indicated by the manufacturers guidelines.

SEM was performed on the sample groups at time points of 1.5 h, one day, and 21 days to visualize the magnetic particles adhered to the osteoblasts. Briefly, at the stated time points, the samples were fixed in 2.5% glutaraldehyde in PBS before rinsing in PBS. The samples were then dehydrated in graded series of ethanol before critical point drying. The samples were then gold sputtered before viewing using a Hitachi scanning electron microscope.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze mRNA produced of bone-related gene osteopontin normalized to GAPDH at 21 days from the different sample groups. This incorporated the use of a Roche lightcycler using SYBRgreen fluorescence. The primers used were those described by Martin *et al.* [19]. Primers were used at a 500-nM concentration and the amplification settings were denaturation at 95 °C for 30 s, a 40-cycle amplification of 95 °C for 0 s, 65 °C for 15 s, 95 °C for 0 s, and a cooling of 40 °C for 30 s.

Von kossa staining for phosphate deposits (mineralized bone matrix) was performed on all six samples at 21 days. The supernatant from each of the wells was removed and the cells were fixed with 70% ethanol for 15–30 min at room temperature. The ethanol was removed, the cells were washed and 1 ml of 5%

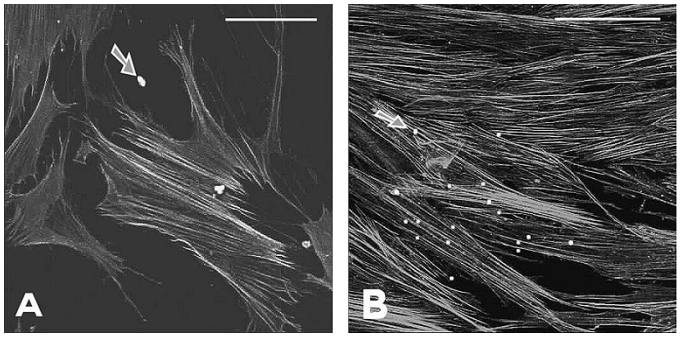


Fig. 2. Laser scanning confocal microscope images of actin filament staining of osteoblasts cultivated for (A) two days and (B) one week in monolayer. Magnetic particles are seen as round white dots as indicated by arrows. Scale bars: (A) 50 $\mu$m and (B) 100 $\mu$m.

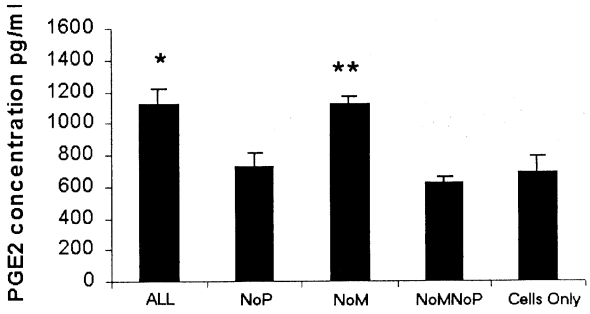


Fig. 3. Prostaglandin $E_2$ ELISA data from three-day culture media. *Group ALL is significantly different from groups NoP, NoMNoP, CellsOnly, and NoCells ($p < 0.05$). **Group NoM is statistically significantly different from groups NoP, NoMNoP, CellsOnly, and NoCells ($p < 0.05$).

silver nitrate solution was added to each well under a fluorescent light for 15–30 min. After washing, 5% sodium thiosulphate solution was added to the wells for 2 min. After a final wash, the samples were left to dry and the samples were viewed using light microscopy.

Data from sample groups were analyzed for statistical significance using a one-way analysis of variance test using the Tukey least significance test for post-hoc comparisons with a significance level of $p < 0.05$.

## III. Results

Fluorescent imaging showed that for sample groups 1)–5) a viable, confluent monolayer of cells was present at 21 days, covering the surface of the wells in the tissue culture plates. Light microscopy of these same samples showed the position of the adhered magnetic particles and clearly showed the absence of the particles in sample groups 2), and 4)–6). It was seen that there were approximately two particles per cell on the cells that had particles adhered in groups 1) and 3). Proliferation of cells during the 21 days had an effect on the average number of particles per cell. Therefore, there were many cells without particles adhered to them in sample groups 1) and 3).

SEM imaging showed confluent cells in monolayer spread out and attached to the surface of the tissue culture well in samples 1)–5) in confirmation of the fluorescent and light microscopy results. Again, no difference was seen in the morphology of the cells between the different sample groups 1)–5).

The LSCM performed at days two and seven showed no significant alteration in the actin filament structure in the osteoblasts between the sample groups (Fig. 2). It was clear that the majority of the particles had been internalized by the cells and that many of these particles appeared to be located close to the nucleus.

ELISA results showed a significant $(p < 0.05)$ upregulation of prostaglandin $E_2$ from group 1) (the experimental group) in comparison to groups 2), 4), 5), and 6) (Fig. 3). There was no significant difference in prostaglandin $E_2$ production between groups 1) and 3).

SEM performed show that the RGD-coated particles had not all been phagocytosed by 1.5 h (Fig. 4) or at the one-day time point. However at 21 days there did not appear to be any particles remaining on the outside of the cells and a confluent layer of cells was seen for sample groups 1)–5) (Fig. 4).

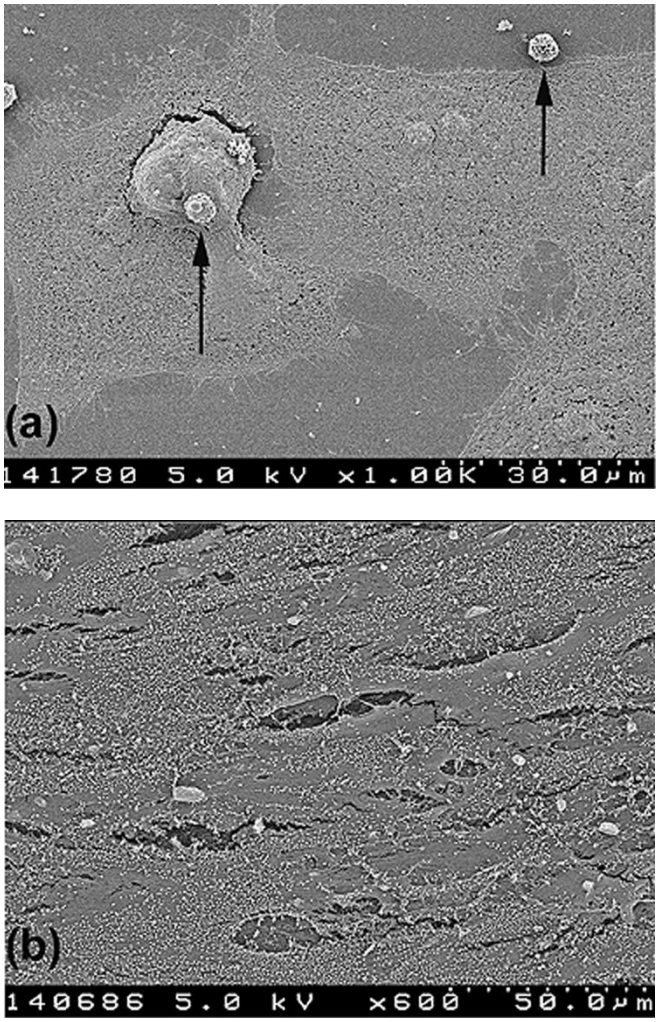


Fig. 4. (a) SEM images (Group 3) RGD-coated magnetic particles after 1.5-h adherence to osteoblast. Round 4.5-micrometer magnetic particles can be clearly seen attached to the spread out cell. This image is typical of groups 1) and 3) at this time point. (b) (Group 3) After 21 days incubation with the osteoblasts, many of the magnetic particles have been internalized and can no longer be seen on the surface of the cells. This image is typical of sample groups 1)–5).

Real-time RT-PCR showed an upregulation of osteopontin in sample group 1) (the experimental group) in comparison to the

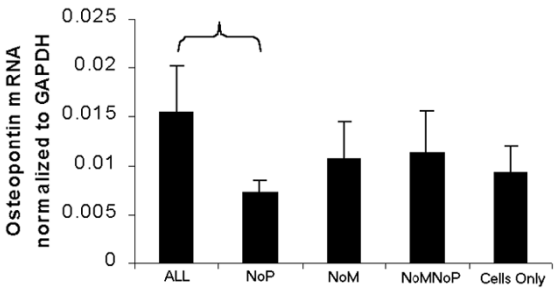


Fig. 5. Real-time RT-PCR data showing osteopontin mRNA levels normalized to GAPDH at 21 days. Groups ALL and NoP are significantly different ($p < 0.05$).

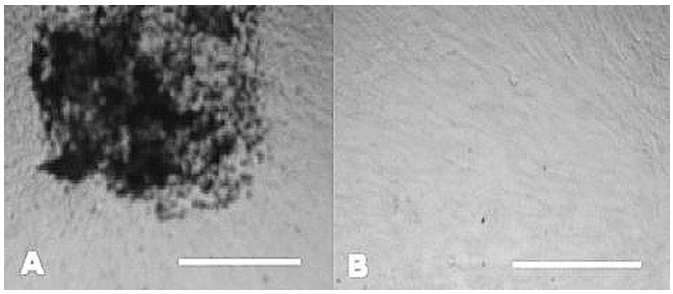


Fig. 6. Light microscopy of von kossa stain at 21 days of sample group 1) (group with particles and applied magnetic field (A). Light microscopy of von kossa stain of sample group 5) -control, (B). Scale $\mathrm{bars} = 50\ \mu$m.

control sample group 2) (sample group consisting of cells with magnetic field but no particles) (Fig. 5). There was no significant difference between the osteopontin mRNA levels expressed between the other sample groups.

Von kossa staining of the wells in sample groups 2)–6), showed no phosphate deposition. However, in sample group 1) (the group containing cells with magnetic particles plus cyclical magnetic field), phosphate staining was present in small amounts (Fig. 6).

## IV. Discussion

The live/dead stain using fluorescent microscopy showed that the presence of the magnetic particles was not causing cell necrosis at 21 days. The actin staining at two days and one week as viewed by confocal microscopy showed little disruption to the cytoskeletal network due to the presence of the particles either in or out of the cyclical magnetic field. The cytoskeleton is capable of sensing and transducing mechanical stress applied to a cell. This signalling is dependant on elastic coupling between the site of applied force and the site where the first biochemical change occurs [20]. Previous studies incorporating mechanical force application using similar magnetic particle technology have seen actin accumulation after continuous 30 min force exposure at the binding sites of the particles to the cell membrane [16]. However, these experiments were performed prior to the particle being phagocytosed (the particle was allowed to adhere for 10 min before force was applied) and may be applying a different magnitude or indeed, a different mechanism of mechanotransduction to the cell than as described in this paper. Phagocytosis itself affects the cytoskeleton by increasing microfilament density.

Engulfment is frequently accompanied by numerous microfilament associations at the point of phagocytosis. At the two-day time point however, no difference in the actin filaments were seen between the different sample groups. Particle internalization is confirmed by SEM analysis.

The migrating of many of the particles to the cell nuclei may be due to the particle size, chemistry or due to the RGD coating. Sethi *et al.* have described that untethered RGD activates integrins, therefore linking with the cytoskeleton but remaining completely free to move under mechanical loads [21]. They suggest that RGD-peptide attaches to an unrestrained integrin ligand that then tends to pull that ligand through the membrane and into the cell. Perhaps the RGD-coated particles, undergoing phagocytosis, may undergo internalization via the same mechanism which may explain the localization of many of the particles at the nuclei of the cells. We are investigating further how the particles are internalised, analysing different coatings of these particles and how this affects the mechanical stimulation applied to the cell via particle movement in the time-varying magnetic field.

It is well accepted that $\mathrm{PGE}_2$ stimulates bone resorption [22]. The increase in $\mathrm{PGE}_2$ production in groups 1) and 3) in comparison to the other groups maybe due to the internalization of the particles that may in turn provide physical stimulation. Phagocytosis of 1–3 $\mu$m sized particles (titanium, cobalt, and polyethylene) by osteoblasts have been noted to upregulate $\mathrm{PGE}_2$ release [23], [24]. Further studies are currently being performed to investigate whether or not the $\mathrm{PGE}_2$ upregulation is solely due to phagocytostis of the magnetic particles or if it also relates to the magnitude of movement of the particles in an applied magnetic field.

Osteopontin—which was upregulated in this study—is believed to play a key role in different steps of differentiation and activity of bone cells and in the maturation and mineralization of bone matrix [25]. It has been described previously that osteopontin is upregulated due to mechanical strain application to osteoblasts [26], [27]. The upregulation of osteopontin mRNA seen in the real-time RT-PCR with experimental sample group 1) in comparison to group 2) may correlate to the same mechanical stimulation response as applied by the moving magnetic particles.

It has been documented in previous experiments that the application of mechanical stress to bone cells induces matrix mineralization production [28]. The small amount of mineralization (as seen by the von kossa stain) in the sample group that had adhered particles plus magnetic field applied (with no mineralization seen in any of the control groups) also may be due to a response to mechanical stimulation from the particles.

In this experiment, we have gone further than previous studies and extended the culture time to 21 days. In this way, we can determine the application of long-term magnetic loading on the culture of these cells. It is difficult to compare the magnitude of the mechanical stress applied in previously published experiments as those stresses were applied to the scaffolds rather than the cell membrane. This highlights a major disadvantage of most conditioning systems—the force must be transmitted from the scaffold to the cells, which are attached to the inside linings of the scaffold pores (where pore diameter is large compared with

cell diameter). One of the advantages of the method described here is that the force is applied directly to the cell rather than relying on transmission of the force from the scaffold to the cell. With optimization, this will allow for the tailored application of piconewton-scale forces to the cell membrane, eliminating the need for mechanically strong scaffold materials.

Initial results indicate that adherence of RGD-coated, 4.5-$\mu$m ferromagnetic particles to primary human osteoblasts does not initiate cell necrosis up to 21 days *in vitro*. Also, though these results are preliminary, mechanical stimulation of primary human osteoblasts by magnetic particle technology appears to have an influence on osteoblastic activity.

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