# Strategies Combining Cells and Scaffolds for Bone Tissue Engineering

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*Abstract*: Engineering bone typically uses highly porous scaffolds, osteoblasts or cells that can become osteoblasts, and regulating factors that promote cell attachment, differentiation, and mineralized bone formation. In this study we investigated the effects of the electromagnetic stimulation on SAOS-2 cells, from a human osteosarcoma cell line using a sintered 3D titanium scaffold. In comparison with control conditions (standard cell culture incubator, where no electromagnetic stimulus was detectable), the electromagnetic stimulus (magnetic field, 2 mT; frequency, 75 Hz) increased the cell proliferation and the surface coating with decorin, osteocalcin, osteopontin, and type-I collagen. The electromagnetic stimulus aimed at obtaining an improved cell proliferation and production of bone proteins, with a consequent surface coating of the scaffold. The protein-coated 3D titanium scaffold could be used, in clinical applications, as an implant for bone repair.

## 1. *Introduction*

A key component in tissue engineering for bone regeneration is the scaffold that serves as a template for cell colonization and deposition of bone extracellular matrix thus providing structural support to the newly formed tissue [1,2].

The use of titanium or titanium alloy (Ti-alloy) as biomaterials is possible because of their very favourable biocompatibility with living tissue [3]. In particular, Ti-alloy implants are widely used in orthopaedics and dentistry because of their good mechanical properties and surface biocompatibility [4]. However, after implantation the lack of complete adherence between Ti-alloy implants and bone tissue is one of the major problems to solve [5].

To enhance bone implant osteointegration, many strategies have been developed, including the optimization of implant material, implant design, surface morphology and osteogenic coatings [6]. Other methods that have been attempted to enhance endogenous bone healing around biomaterials are different forms of biophysical stimulations such as pulsed electromagnetic fields (PEMFs) and low intensity pulsed ultrasounds (LIPUS), which were initially developed to accelerate fracture healing [7,8].

Studies of electric and electromagnetic fields suggest they (1) regulate proteoglycan and collagen synthesis and increase bone formation in models of endochondral ossification, (2) accelerate bone formation and repair, (3) increase union rates in fractures previously refractory to healing, and (4) produce results

equivalent to bone grafts. Investigations have begun to clarify how cells respond to biophysical stimuli by means of transmembrane signaling and gene expression for structural and signaling proteins [9-11]. In our previous *in vitro* studies, we successfully investigated the effect of the electromagnetic stimulation on SAOS-2 human osteoblast proliferation and calcified matrix production using porous hydrophobic cross-linked polyurethane [12,13].
The overall objective of this research is to investigate the effects due to the application of a pulsed electromagnetic wave onto a rough and macroporous sintered 3D titanium scaffold cultured with SAOS-2 cells: using this approach, the protein-coated titanium 3D scaffold may be used, in clinical applications, as an implant for bone repair in order to enhance the *in vivo* osteointegration process.

## 2. *Materials and Methods*

### 2.1 *Ti-alloy 3D scaffolds*

Three-dimensional scaffolds (disks) (diameter, 12 mm; height, 4 mm) were obtained by sintering a powder (average diameter, 40 μm) of titanium alloy Ti6Al4V (ISO 5832-3) at 1100°C for 2.5 h. After the sintering process the relative density was around 60%.These scaffolds were provided by Lima Lto (Villanova di San Daniele del Friuli, Udine, Italy).

### 2.2 *Cell seeding*

The human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (HTB85, ATCC). The cells were cultured in McCoy's 5A modified medium with L-glutamine and HEPES (Cambrex BioScience), supplemented with 15% fetal bovine serum, 2% sodium pyruvate, 1% antibiotics, $10^{-8}$ M dexamethasone, and 10 mM $\beta$-glycerophosphate (Sigma-Aldrich). The cells were cultured at 37°C with 5% $CO_2$.
The disks were sterilized by ethylene oxide at 38°C for 8 h at 65% relative humidity. After 24 h of aeration in order to remove the residual ethylene oxide, the disks were placed inside the two culture systems: the "static" one, a standard well-plate system, far from the electromagnetic bioreactor, and the "electromagnetic" one, where the well-plate system is positioned inside the electromagnetic bioreactor [12,13]. A cell suspension of $4\times10^5$ cells in 100 μl was added onto the top of each disk and, after 0.5 h, 1 ml of culture medium was added to cover the disks. Cells were allowed to attach overnight, then the static culture continued in the standard well-plate, and the electromagnetic bioreactor was turned on.

### 2.3 *Physical stimulus and control culture*

The electromagnetic bioreactor [12,13] consisted of a carrying structure machined in a polymethylmethacrylate tube: the windowed tube carried a well-plate and two solenoids, the planes of whom were parallel. The disk surfaces were 5 cm distant from each solenoid plane, and the solenoids were powered by a Biostim SPT pulse generator (Igea, Carpi, Italy), a generator of Pulsed Electromagnetic Fields (PEMFs). Given the position of the solenoids and the characteristics of the pulse generator, the electromagnetic stimulation had the fol-

lowing parameters: intensity of the magnetic field equal to 2 ± 0.2 mT, amplitude of the induced electric tension equal to 5 ± 1 mV, signal frequency of 75 ± 2 Hz, and pulse duration of about 1.3 ms. The electromagnetic bioreactor was placed into a standard cell culture incubator with an environment of 37°C and 5% $CO_2$. The electromagnetic culture was stimulated by the PEMF 24 h per day for a total of 22 days. The control or static culture was incubated for the same period of time in a different $CO_2$ incubator. The culture medium was changed in the three different culture systems on days 4, 7, 10, 13, 16, and 19.

2.4 *Scanning electron microscopy (SEM) analysis*
The disks were fixed with 2.5% (v/v) glutaraldehyde solution in 0.1 M Na-cacodylate buffer (pH=7.2) for 1 h at 4°C, washed with Na-cacodylate buffer, and then dehydrated at room temperature in a gradient ethanol series up to 100%. The samples were kept in 100% ethanol for 15 min, and then critical point-dried with $CO_2$. The specimens were mounted on aluminum stubs, sputter coated with gold (degree of purity equal to 99%), and then observed with a Leica Cambridge Stereoscan 440 microscope at 8 kV.

2.5 *DNA content*
Cells were lysed by a freeze-thaw method in sterile deionized distilled water. The released DNA content was evaluated with a fluorometric method (PicoGreen, Molecular Probes). A DNA standard curve, obtained from a known amount of osteoblasts, was used to express the results as cell number per disk.

2.6 *Rabbit polyclonal antiserum and purified antigen*
The rabbit polyclonal antibody IgG anti-type-I collagen was kindly provided by Dr. Fisher (http://csdb.nidcr.nih.gov/csdb/antisera.htm, National Institutes of Health, Bethesda, MD). The antigen type-I collagen was purified as previously described [14].

2.7 *Immunofluorescence staining*
At the end of the culture period, the disks were fixed with 4% (w/v) paraformaldehyde solution in 0.1 M phosphate buffer (pH=7.4) for 8 h at room temperature and washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, pH=7.4) three times for 15 min. The disks were then blocked by incubating with PAT (PBS containing 1% [w/v] bovine serum albumin and 0.02% [v/v] Tween 20) for 2 h at room temperature and washed. Anti-type-I collagen rabbit polyclonal antiserum was used as primary antibody with a dilution equal to 1:1000 in PAT. The incubation with the primary antibody was performed overnight at 4°C, whereas the negative control was based upon the incubation, overnight at 4°C, with PAT instead of the primary antibody. The disks and the negative control were washed and incubated with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes) with a dilution of 1:500 in PAT for 1 h at room temperature. The disks were then washed in PBS, counterstained with a solution of propidium iodide (2 μg/ml) to target the cellu-

lar nuclei, and washed. Images were taken with a fluorescence microscope. The fluorescence background of the negative control was almost negligible.

2.8 *Extraction of the bone matrix*

At the end of the culture period, the cultured disks were washed extensively with sterile PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, pH=7.4) in order to remove the culture medium, and then incubated for 24 h at 37°C with 1 ml of sterile sample buffer (1.5 M Tris-HCl, 60% [w/v] sucrose, 0.8% [w/v] Na-dodecylsulphate, pH=8.0). At the end of the incubation period, the sample buffer aliquots were removed and the total protein concentration in the three culture systems was evaluated by the BCA Protein Assay Kit (Pierce Biotechnology). The total protein concentration was 106 ± 12 μg/ml in the static culture, and 150 ± 14 μg/ml in the electromagnetic culture ($p<0.05$ in the comparisons "static vs. electromagnetic"). The calibration curve to measure type-I collagen was performed by an ELISA assay [12,13]. The results are expressed as fg/(cell×disks).

2.9 *Statistics*

Results are expressed as mean ± standard deviation. In order to compare the results between the culture systems, one-way analysis of variance (ANOVA) with *post hoc* Bonferroni test was applied, electing a significance level of 0.05.

3. *Results*

The human SAOS-2 osteoblasts were seeded onto the surface of sintered titanium 3D scaffolds, and then cultured without or with an electromagnetic stimulus for 22 days. These culture methods allowed to study and compare the difference in the cell-matrix deposition and distribution between the two culture systems.

3.1 *Microscope analysis*

Using SEM observation, the scaffold morphology appeared composed of a three-dimensional mesh showing regular square and interconnected holes with side of 800 μm (Fig. 1A). Ti surfaces were quite uniform and smooth as shown in Fig. 1B.

After incubation with SAOS-2 cells, SEM images revealed that, because of the electromagnetic stimulation, the cells proliferated over the available surface of the 3D titanium scaffolds (Fig. 2B); statically cultured cells were few and were essentially organized in a monolayer (Fig. 2A).

These observations were confirmed by the measure of the DNA content after 22 days of culture: in the static culture the cell number per 3D scaffold grew to $32.8\times10^6 \pm 8.2\times10^4$ and in the electromagnetic culture to $47.5\times10^6 \pm 8.4\times10^4$ with $p<0.05$.

The immunolocalization of type-I collagen showed a more intense fluorescence in the electromagnetically cultured disk than in the static condition, revealing that stimulation is effective in terms of higher cell proliferation and more intense production of the extracellular matrix (Figs. 3A and 3B). The immunolo-

calization of decorin, osteopontin, and osteocalcin confirmed a similar culture structure (data not shown).

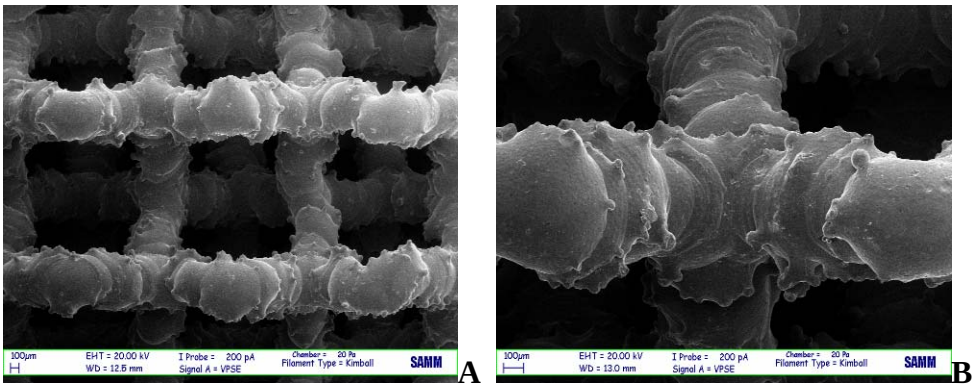


Fig. 1. Scanning electron microscopy observation of an unseeded 3D titanium scaffold at 25× magnification (A) and at 100× magnification (B).

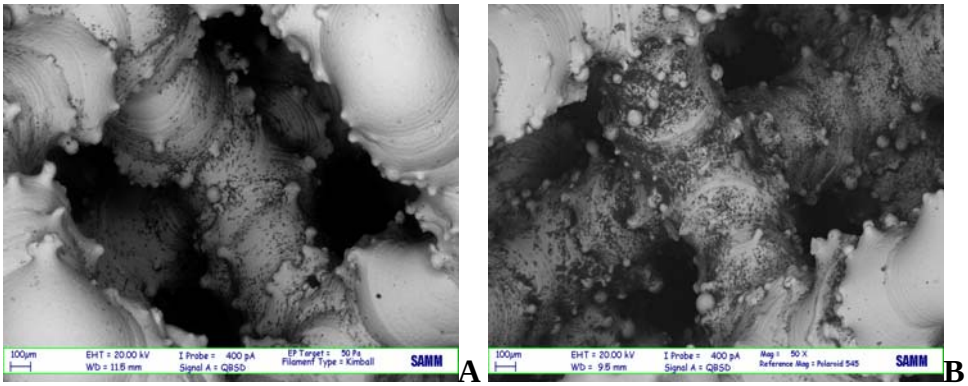


Fig. 2. Scanning electron microscopy observation of a seeded 3D titanium scaffold at 50× magnification in static (A) and dynamic culture condition (B).

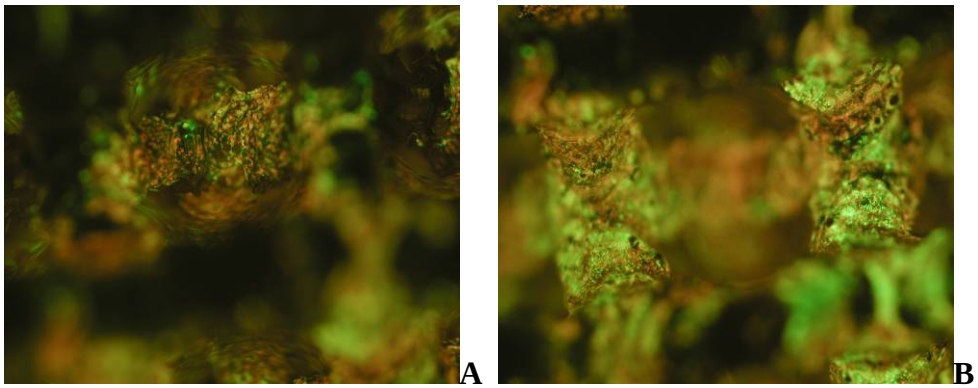


Fig. 3. Immunolocalization of type-I collagen (green) in the static (A) and electromagnetic (B) cultures.

### *3.2. Extracellular matrix extraction*

In order to evaluate the amount of bone matrix over the 3D titanium scaffold surface, an ELISA of the extracted matrix was performed: at the end of the culture period, in

comparison with the static culture, the electromagnetic stimulus increased the coating with decorin, osteopontin, osteocalcin, type-I and type-III collagen ($p<0.05$) (Table 1).

Table 1. Amount of extracellular matrix constituents inside 3D scaffolds.

| | Matrix total coating after 22 days of culture in fg/(cell x disks) | | |
|---|---|---|---|
| | Static culture | Electromagnetic culture | Electromagnetic/Static |
| Decorin | 6.31 ± 0.11 | 11.8 ± 0.11 | 1.87-fold |
| Osteocalcin | 3.53 ± 0.12 | 11.2 ± 0.06 | 3.2-fold |
| Osteopontin | 4.7 ± 0.09 | 5.69 ± 0.07 | 1.20-fold |
| Type-I coll. | 48.1 ± 0.10 | 196 ± 0.20 | 4.07-fold |
| Type-III coll. | 18.7 ± 0.08 | 33.9 ± 0.14 | 1.81-fold |

Table note: $p<0.05$ in all "Static" vs. "Electromagnetic" comparisons.

## 4. *Discussion*

In this *in vitro* study we have shown the effects due to the application of a pulsed electromagnetic wave onto a rough and macroporous sintered 3D titanium scaffold cultured with SAOS-2 cells.

The electromagnetic wave enhancing cell adhesion and proliferation, with consequent production of ECM proteins, should behave as a coating of the biomaterial surface [12,13,15]. The results obtained in this study indicate that the electromagnetic stimulus increased the cell proliferation around 1.5-fold. Furthermore, as shown by an ELISA assay and immunolocalization experiments, the electromagnetic field significantly enhanced the synthesis of type-I collagen, decorin, osteopontin, osteocalcin, and type-III collagen, which are fundamental constituents of the physiological bone matrix. PEMFs are known to increase osteoblastic proliferation, extracellular matrix production, and insulin growth factor-2 (IGF-2) production [16,17]. Although PEMF treatment is effective *in vivo* [18], the molecular and cellular mechanisms underlying the responsiveness of cells and tissues to PEMFs are yet to be identified. The physical-chemical interactions between biological tissues and PEMF may occur outside the cell and then propagate and amplify through conventional or novel signal transduction pathways. A stimulation of transduction pathways is apparent by PEMF, resulting in increased cytosolic $Ca^{2+}$ and activation of calmodulin, which finally stimulate osteoblastic cell proliferation [19]. Recently, PEMF was shown to rapidly activate the mTOR signaling pathway, suggesting that PEMF exposure might function in a manner analogous to soluble growth factors [20].

In the model we presented, SAOS-2 cells were used because they are relatively easy to maintain and are a well-characterized osteosarcoma human cell line; in order to obtain a tissue-engineering product for bone repair a better result could be reached using autologous bone marrow stromal cells because they do not elicit the immunitary response of the patient upon implantation. In conclusion, electromagnetic stimulus of cell cultures is a promising tool for stimulating cell proliferation and activity and may ultimately be used to speed up growth of engineered tissues for implantation *in vitro*.

## 5. *Acknowledgments*

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