NMP3-CT-2005-013912

SmartCap

Injectable macroporous biomaterial based on Calcium Phosphate cement for bone regeneration

SPECIFIC TARGETED RESEARCH PROJECT

Priority 3 - NMP

Publishable final activity report

Period covered: from 1–05-2007 to 31-08-2008 **Date of preparation**: 15-10-2008

Start date of project: 1-05-2005 **Duration:** 40 months

Project coordinator: Prof. Josep A. Planell
Project coordinator organisation: Technical University of Catalonia (UPC)

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1. Project execution*

Project objectives

Osteoporosis and bone degeneration in aging populations as well as bone defects caused by trauma and pathology grounds a societal need for therapeutic products. The main goal and the breakthrough of this project are to implement a novel concept of biomaterials for bone regeneration, with a range of properties that elicit specific cell responses. The biomaterials developed in this project will give improvements in health, quality of life, environment and safety. These multifunctional biomaterials will be injectable, porous, intelligent and biodegradable to promote osteogenesis and angiogenesis. Because these biomaterials will be injectable, they will be applied by means of minimally invasive surgery. This means less suffering by the patient, reduction of health care cost, simple surgical technique and improved working conditions. These biomaterials will be an advantageous alternative to autologous bone due to their large availability and that they can be obtained sterilized right off the shelf.

Contractors involved

UNIVERSITAT POLITECNICA DE CATALUNYA
having its registered office at :
Jordi Girona 31
08034 Barcelona (Spain)
Hereinafter referred to as "UPC"

CONSIGLIO NAZIONALE DELLE RICERCHE
having its registered office at :
Piazzale Aldo Moro 7
00185 Roma (Italia)
Hereinafter referred to as "CNR-IMCB"

ISTITUTI ORTOPEDICI RIZZOLI
having its registered office at :
Via di Barbiano 1/10
40136 Bologna (Italia)
Hereinafter referred to as "IOR"

UNIVERSITY OF BRIGHTON
having its registered office at :
Mithras House, Lewes Road
BN2 4AT Brighton (United Kingdom)
Hereinafter referred to as "UoB"

* Since a patent about the materials developed within the project is being yet developed, in this report we only present general results in order not to jeopardize the patent process.

PROVOST, FELLOWS, AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNDIVIDED TRINITY OF QUEEN ELIZABETH NEAR DUBLIN
having its registered office at :
University of Dublin, College Green
Dublin 2 (Ireland)
Hereinafter referred to as "TCD"

UNIVERSITY OF GHENT
having its registered office at :
St. Pietersnieuwstraat 25
B 9000 Ghent (Belgium)
Hereinafter referred to as "UG"

UNIVERSITAET ULM
having its registered office at :
Albert-Einstein-Allee 7 (Project Postal Albert-Einstein-Allee, 29)
89081 Ulm (Germany)
Hereinafter referred to as "UoU"

Co-ordinator contact details:

Prof. Josep A. Planell
Department of Material Sciences and Metallurgical Engineering
Technical University of Catalonia
Diagonal 647,
08028 Barcelona
Spain
Tel: +34 934011612
Fax: +34 934016706
Email: josep.a.planell@upc.es

Expected end results

The main goal of this project consists in the implementation of the concept of "intelligent" injectable self-setting biodegradable macro-micro porous biomaterials, promoting angiogenesis and osteogenesis, for bone regeneration.

Intentions for use

These "intelligent" biomaterials are intented to solve problems related to osteoporosis, bone tumours, spinal disorders, severe trauma to the extremities, crippling diseases, deformities in children and fracture healing, affecting hundreds of thousands of people (according to Bone and Joint Decade) in Europe and worldwide.

Impact

The project will have impact on European quality of life by:

- Raising the number of patients who can be treated
- Reducing the costs related with bone treatments with increasing quality
- Introducing new therapeutic substances such as growth factors and differentiation agents
- Generating new processing methodologies that can be used on other types of industries
- Generating high level knowledge that will be used by the different high education institutions to teach their students
- Reducing patient discomfort in all aspects
- Creating new job positions directly and indirectly related to the project
- Widening the market for European companies in the field of biomaterials for tissue replacement and regeneration systems

Work performed and results

The SmartCaP project was conceived to introduce a novel concept of biomaterials for bone repair and regeneration. These biomaterials should be multifunctional, injectable, self-setting, macroporous, "intelligent", biodegradable, easy to apply and sterilizable.
To respond to all these requirements, the project was divided in 7 workpackages (plus one regarding management issues).

The following work has been performed:

WP 1: Material design, fabrication and characterization

The main objectives of this workpackage were to modify chemically a calcium phosphate (CaP) bone cement to make it self-setting, intelligent, injectable, and multiporous (macro, micro and nano) and to characterize it.

Taking into account the results obtained, a two-fold approach has been followed, selecting, in addition to the injectable materials, another group of materials which are not injectable immediately after being mixed, but which can be more appropriate for in vitro tissue engineering applications. Thus, two families of materials were selected:

a) Materials that can be injected in vivo, which are macroporous and have good cohesion: they can be injected immediately after preparation. Three formulations and a control have been proposed.

b) Materials for scaffolds, which have a good interconnected macroporosity, and therefore can be very adequate for bioreactor studies. They can be used as pre-set low temperature scaffolds. Three formulations and a control have been proposed

In addition, these resulting materials have been characterised in terms of:

- Setting reaction
- Porosity
- Injectability
- Mechanical properties
- Setting reaction kinetics
- Specific surface area
- Microstructure

WP 2: Functionalisation for improved osteogenesis and angiogenesis

Biological and chemical modifications of the biomaterials were performed in order to enhance bioactivity. An improvement in cell attachment to the material through integrins was used to modulate cell signal transduction cascade regulating gene expression.

For the functionalization, 4 peptides were selected relevant to osteogenesis. Their sequences have been designed to offer the best possible exposure of the relevant bioligands to osteoblasts

Their combined use in biomaterial surface functionalisation has been shown to produce a synergistic effect by favouring both osteoblast migration and adhesion. These peptides were prepared using standard methods for peptide synthesis. All peptides were analysed by liquid chromatography and mass spectrometry.

After the functionalization with peptides, the interaction between human MSCs and the functionalised composites was studied. Functionalised composites were prepared in sterile conditions under ventilated hood. Since the pre-wetting of the composites with the culture medium containing 10% fetal bovine serum leads to protein adsorption over the surfaces, and this could mask the effect of the composite functionalisation, human MSCs were seeded on the composites in serum-free media.MSC-seeded composites were cultured up to 72h, and cell adhesion, spreading, viability, growth and death were measured.
No difference in cell behaviour on functionalised or nonfunctionalised composites was found, at the concentrations studied.

WP 3: Biological Molecules loading and delivery assays

Materials developed in WP 1 were loaded with growth factors and drugs. Liberation studies were performed in order to assess the mid-term release of the biological entities.

In this WP, the research performed was focused on the following tasks:

- Integration/encapsulation of viable molecules:
  - VEGF (VEGF analogue)
  - BMP (BMP analogue)
  - Anti-inflammatory drugs
- Delivery and release of molecules

- In vitro evaluation of molecule release
    - Endothelial cells
    - Osteoblasts
    - Monocytes/macrophages activation

We have been able to assess in vitro the potential of the candidate materials to deliver bioactive agents in a controlled and effective manner and to prove that all the cements selected showed to be able to stimulate relevant cells towards proliferation.

WP 4: Isolation and culture of primary cells

Isolation and expansion of primary osteoblasts, osteogenic progenitor cells, osteoclasts from human tissue and endothelial cells from immortal cell line were performed and seeded on materials. Mesenchymal cells from bone marrow were induced to differentiate into osteoblasts, and seeded onto materials. Morphology, biochemistry, and bone forming ability of cells interacting with materials will be analyzed. Gene expression of the cell/material construct was also analyzed.

In the development of new materials for bone regeneration, the biological assays provide meaningful results which aid in the design of biomimetic scaffolds.

For the screening of samples osteoblast-like Saos-2 cells have been used, because of the advantage in terms of availability (of cells), high proliferation rate and repeatability (of results). The materials were pre-treated with serum-added culture medium, according to an approach as close as possible to the in vivo events following implantation, when body fluids immediately coat the foreign surface. The composites tested showed no toxicity and allowed cell adhesion (Figure IOR-1) and some proliferation.

Subsequently, primary cells involved in bone regeneration were used to obtain meaningful and specific results on cell reaction to any chemical/topographical change of the surface. Primary cells, such as bone marrow stromal cells, osteoblasts, and endothelial cells were isolated, expanded and characterized, and their interaction with the selected composites was studied. Human osteoblasts from trabecular bone (HOB) are differentiated cells which usually in vivo face the implant surface, therefore their response is highly significant. Using HOB it was found that γ-ray treatment of final scaffolds induced some changes in the material structure, which in turn affected the response of cells. This is a remarkable point not to be underscored, since the scaffolds need to be sterile before implantation. Therefore, the composites were prepared starting from sterile raw powders (γ-irradiation), in sterile condition under ventilated hood.

Bone marrow stromal cells (BMSC) are the main cells involved in bone regeneration, through differentiation to tissue-specific lineage and secretory activity at the site of bone repair.

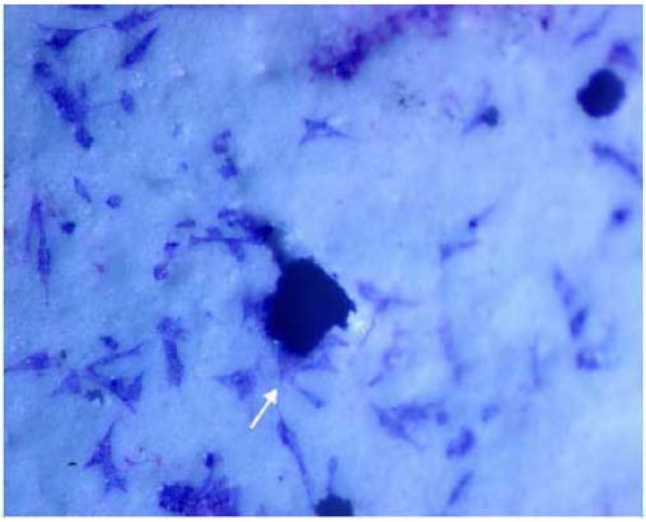

Figure 1: Saos-2 cells morphology on composites at 72h. A few cells appeared partially immersed in the cement bulk (white arrow) (Light microscopy, x30).

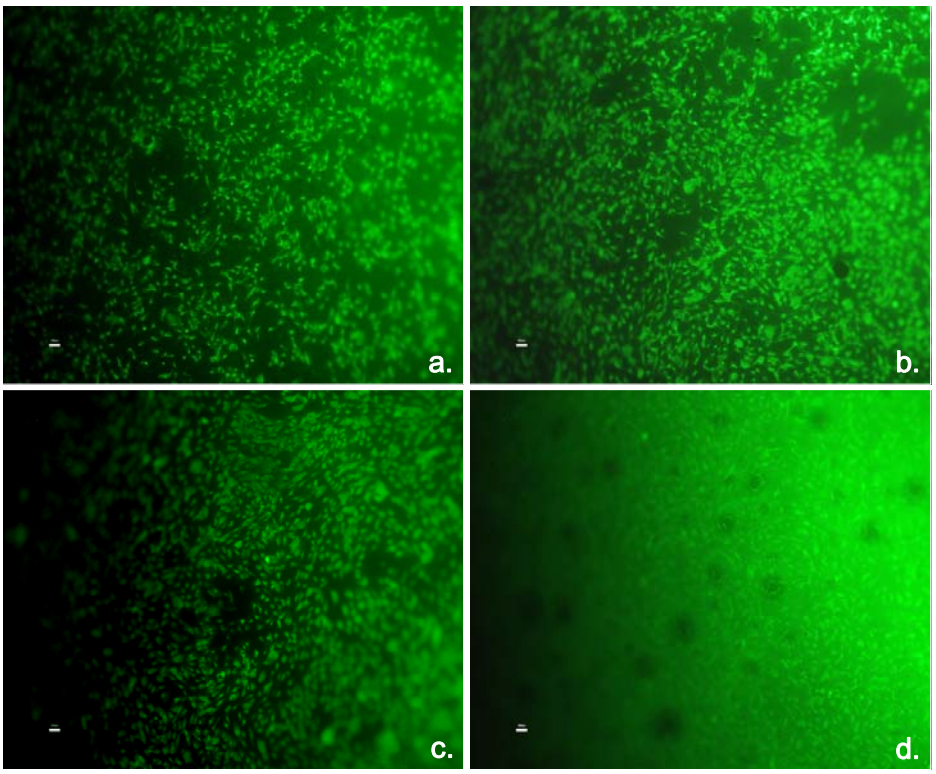

Figure 2: Cell spreading (72 h) using orange acridine staining (x4 magnification) on selected composites

WP 5: In vitro effect of mechanical stimuli on biomaterial biological performance

The effect of mechanical stimuli when applied on the scaffold in a bioreactor has been investigated. The materials developed were tested in conditions that mimic as closely as possible the in vivo conditions. Moreover, the release of ions during cell proliferation and differentiation was monitored in situ through biosensors.

We designed a model for testing the effect of mechanical forces on the biological behaviour of rat mesenchymal stem cells (rMSCs). We have determined the *in vitro* effects of the foamed CaP scaffolds on these cells, in terms of proliferation and differentiation. We seeded 500.000 cells in 6 mm long scaffolds in the perfusion chamber for 2 hours at 10 mm/s fluid flow. Results (see figure below) -obtained by LDH assay and fluorescent microscopic analysis of the samples- showed a good cell distribution and survival, with an approximate seeding efficiency of 35%. Next, we established conditions for dynamic culturing for 3 and 7 days at a fluid flow rate of 1 mm/s. Results showed high proliferation rates, even distributions and increasing numbers of cells over the different time periods. Even more, cells were starting to associate to create tissue-like assemblies covering the surfaces of the scaffold -more in-depth research is planned at this point-. This was somehow unexpected since in *in vitro* static culture there is a lag phase of cell adaptation to the CaP. We believe a combination of factors, like fluid flow dissipation of strong ionic gradients and mechanical loading could be improving the biological performance of the material, which is more in agreement with *in vivo* observations.

Extracellular matrix production was evaluated measuring osteocalcin by confocal microscopy after 14 days of culture. Cells grew inside the macropores, and reached confluence conditions. Furthermore, they were producing osteocalcin, a differentiation marker for osteoblastic precursor cells, without adding osteogenic factors to the cell culture media. The overlapping of images at different focal depth position allowed to recognize cells, spread outside and inside the macropores, due to their higher fluorescence compared to the substrate.

Related to biosensors, a K^+ ISFET has been developed to measure cell death. Moreover, a decrease in Ca^{2+} signalling due to mineralization has been taken as a late maturation marker for bone regeneration. Sensors based on AgCl wires are being developed to selectively detect Ca^{2+} concentrations on a bioreactor system.

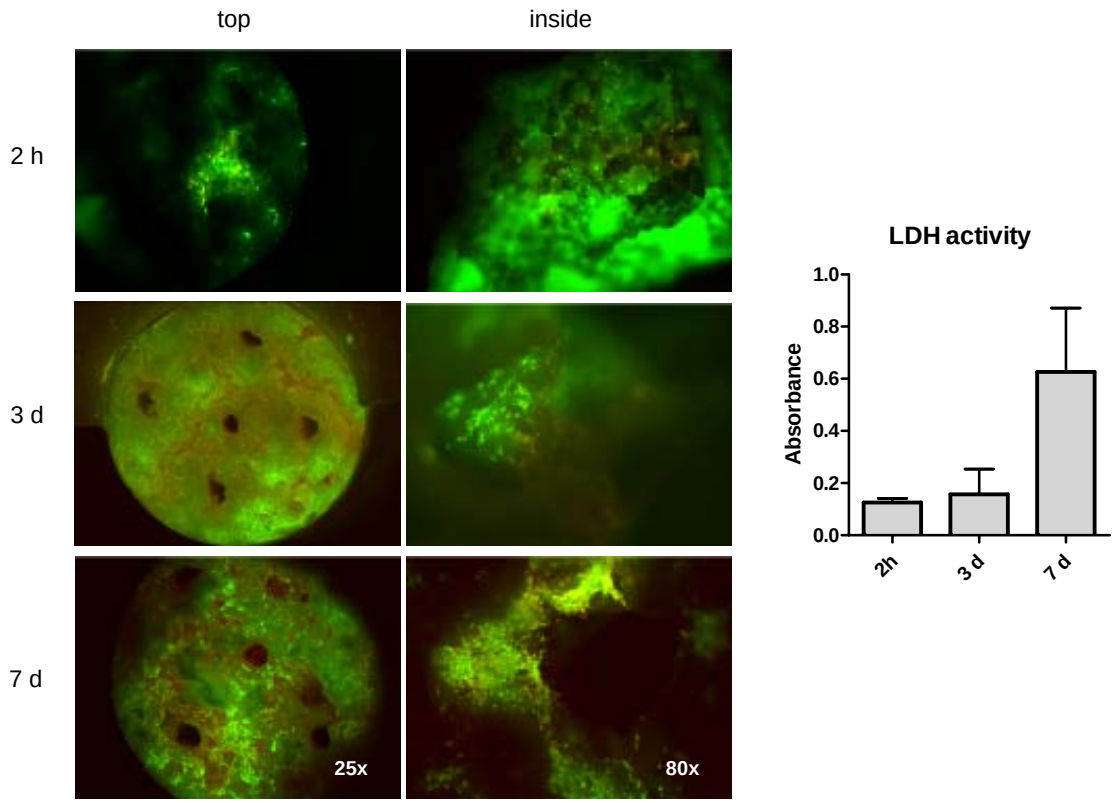

Figure 3: Biomaterial biological performance

WP 6: Computer simulations of bone regeneration

Throughout the project, this workpackage has been used in parallel with the experimental tasks in order to give complementary to the experimental results and to get a different approach of implementing the proposed strategy. Computer models were used to help designing the mechanical requirements of the biomaterials developed in WP1. Computer simulations were performed to model the release over time of growth factors and drug delivery. The interaction of the mechanical stimuli with the biological processes on the biomaterials was analysed through a mechanoregulation model. Moreover, a device for injection was designed and fabricated for all materials.

A computational model for tissue differentiation inside a regular structured porous scaffold has been developed. The model simulates individual cell activity as a response to the mechanical environment and nutrient supply. The computational simulation combines a 3D stochastic model for cell migration and proliferation with a mechano-regulation model for cell differentiation. Cell positions are defined in terms of a regular lattice (a 3D grid) inside the scaffold, where each lattice point represents a possible position for a cell to occupy. Capillary growth is modelled through the migration and proliferation of endothelial cell at the capillary tip, regulated by the chemical and mechanical environments in the vicinity. Simulations predicted a dense network developed at the edges of the scaffold, whereas no vessel formation was predicted in the deeper regions. Endothelial cells did not penetrate further than two milimeters and proliferated at a very small rate after seven days. Bone was predicted in areas surrounding blood vessels while cartilage was predicted in the regions close to the

surface (due to the high levels of fluid flow) and in the core of the scaffold (due to the lack of capillaries and the associated lack of oxygen concentration). Higher loading conditions resulted in higher amounts of cartilage and a less developed vasculature. These results offer a guideline to the design of porous constructs.

WP 7: In vivo biomaterials evaluation: investigation of biocompatibility and osseointegration

Following the results obtained in WP4 and WP5, selected biomaterials were implanted in vivo in rabbits. The performance of the biomaterials was evaluated at month 1, 3, and 5. This workpackage has served as a prototype study that demonstrate the feasibility of the projected breakthrough.

Following the results obtained in WP4 and WP 5, the selected 4 biomaterials were investigated in vivo by implanting them in drill hole defects in femoral condyles of rabbits. Additionally, the materials were implanted subcutaneously. Qualitative and quantitative histological evaluation, biomechanical testing and pQCT-analysis were performed after 1, 3 and 5 months of implantation. In general the biocompatibility of all materials was good. No pronounced inflammatory reactions were observed with any of the tested materials. All materials had a high porosity with some interconnectivity. In most cases cells and tissue infiltrated only pores close to the interface.

The material treated defect region exhibited an increased stiffness at each implantation time point compared to the same region of healthy femoral condyles. The density in the materials treated defect region was also increased at each implantation time point compared to that of healthy femoral condyles.

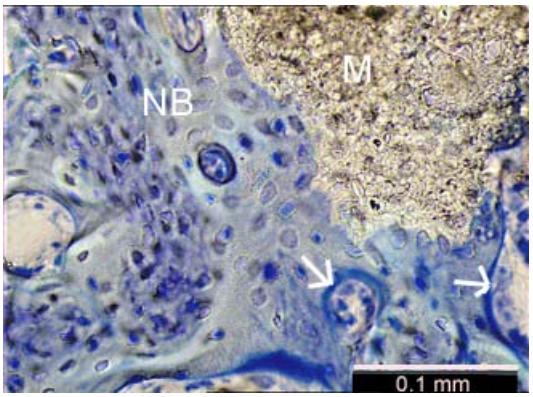

Figure 4: Histology of one of the materials after 1 month of implantation: close material (M) to bone contact (NB = newly formed bone); arrows mark osteoblasts forming new bone; bar = 0.1mm

Summary of results

Composite materials based on Calcium Phosphate cements have been functionalised to improve cell adhesion and seeded with growth factors and drugs to make them multifunctional. Moreover, an injecting device has been designed to improve the minimally invasive surgical technique.
In parallel, computer models have been developed to optimize the biomaterial and obtain a better understanding of the interactions between mechanical and biological processes.
The resulting biomaterials have been tested in vivo, showing their regeneration function.
A patent is being prepared as a previous step to the commercialization of these biomaterials.

2. Dissemination and use

Unfortunately the preparation of the patent has taken a long time given that there are four institutions involved and that we wanted to include the beneficial effect of the biomaterials shown in the in vivo experiments, which ended by the end of the project. This fact has so far hindered the dissemination of the project.
Nevertheless, we have indeed performed several dissemination activities:

1. Set up and updating of project website: www.smartcap.eu
2. Publications of three issues of Project Newsletter (www.smartcap.eu/newsletter)
3. Design of a logo of the project
4. Conferences

- Bioengineering in Ireland Conference, 27/28th of January 2006, Galway (Ireland). Title: "Effect of Scaffold Porosity on Bone Regeneration
- 5th World Congress of Biomechanics, 29th July – 4th August 2006, Munich (Germany). Title: "Optimisation of Bone Scaffold Porosity using mechano-biological simulations" -TCD
- 20th European Conference on Biomaterials, 27th September – 1st October 2006, Nantes (France). Title: "Optimisation of Sterilisation Conditions of Biodegradable, Bioactive Soybean-Based Biomaterials". Authors: Standen G, Salvage J, Dedi, C, Sanginario V, Nicolais L, Ambrosio L, Santin M – UoB and IMCB
- Soy and Health Conference 2006, 12-13th October 2006, Düsseldorf (Germany). Title: "Optimisation of Sterilisation Conditions of Biodegradable, Bioactive Soybean-Based Biomaterials". Authors: Santin M, Standen G, Salvage J, Guildford A, Barbosa J, Barbosa M, Merolli A, Sanginario V, Nicolais L, Ambrosio L – UoB and IMCB

- 13th Annual Conference of Bioengineering in Ireland, 26-27 January 2007, Fermanagh (Ireland). Title: "Simulations of bone regeneration as a function of dissolution rate in a regular structured scaffold". Authors: Byrne D P, Kelly D J, Prendergast P J. - TCD
- PMI 2008, 3rd International Conference on Polymer and Moulds Innovation, 17-19 September 2008, Ghent Belgium. Title: "Design, preparation and characterisation of 3D morphologically controlled structures for tissue engineering". Authors : R. De santis, A. Gloria, L. Ambrosio- IMCB
- Tissue Engineering and Regenerative Medicine International Society European Chapter Meeting 2007, 3-7 September 2007, London. Title: "Cyclic tensile strain influences the chondrogenic differentiation of mesenchymal stem cells via stretch-activated ion channels". Authors: Prendergast PJ, Byrne DP, McMahon LA, Campbell VA – TCD
- 3rd International Conference on Computational Bioengineering, 17-19 September 2007, Venezuela. Title: "The effect of loading on bone regeneration in a regular structured scaffold". Authors: D.P. Byrne, D. Lacroix, D.J. Kelly & P.J. Prendergast – TCD
- Bioengineering in Ireland, 25-26 January 2008, Sligo (Ireland). Title: "Comparing random-walk and diffusion methods to simulate cell dispersal within the fracture callus". Authors: Byrne, D.P., Kelly, D.J., Prendergast, P.J.- TCD
- 2007 Summer Workshop of the European Society of Biomechanics on Finite Element Modelling in Biomechanics and Mechanobiology, 30-31 August 2007, Dublin, Ireland. Title: "Finite element modelling of dissolution of a regular structured scaffold for tissue engineering". Authors: Byrne, D.P., Lacroix, D., Kelly, D.J. & Prendergast, P.J - TCD.
- 16th Conference of the European Society of Biomechanics, 6-9 July 2008, Lucerne, Switzerland. Title: "Modelling cell dispersal in the fracture callus". Authors: Byrne, D.P., Lacroix, D., Kelly, D.J. & Prendergast, P.J. - TCD
- 8th World Congress of Biomaterials, 28 May – 1 June 2008, Amsterdam, The Netherlands. Title: "Mechanobiological models in tissue engineering: current concepts and new developments". Authors: Patrick J. Prendergast, Sara Checa, Damien P. Byrne, Josep Planell, Damien Lacroix - TCD
- 16th congress of the European Society of Biomechanics, 6 - 9 July 2008, Lucerne, Switzerland. Title: "Micro CT based FE models of perfusion fluid flow into scaffolds for bone tissue engineering". Authors: C. Sandino, J.A. Planell, D.Lacroix – UPC
- 7th International Symposium on Frontiers in Biomedical Polymers, 24/06-27/06/2007, Gent, Belgium. Title: "Soybean-Based Biomaterials: Preparation Methods & Tissue Regeneration Potential". Authors Matteo Santin, Guy Standen, Jonathan Salvage Luigi Nicolais, Luigi Ambrosio - UoB
- European Technology Platform for Nanotechnology, 25-26 September 2008, Madrid, Spain. Title: "Strategies in Regenerative Medicine; Integrating Materials Science with Biology". Authors: Matteo Santin - UoB

5. Publications:

- *The Soybean Isoflavone Genistein Induces Differentiation of MG63 Human Osteosarcoma Osteoblasts*, Christopher Morris, Julian Thorpe, Luigi Ambrosio, Matteo Santin. Journal of Nutrition, May 2006, Vol. 136, Iss. 5; pg. 1166

- *Calcium phosphate cements: Competitive drug carriers for the musculoskeletal system?,* Maria Pau Ginebra, Tania Traykova, Josep A. Planell. Biomaterials, April 2006 (10): pg. 2171-2177

- *Simulation of tissue differentiation in a scaffold as a function of porosity, Young's modulus, and dissolution rate of a scaffold: application of mechanobiological models in tissue engineering.* D.P. Byrne, D. Lacroix, D.J. Kelly, J. Planell & P.J. Prendergas. Biomaterials 28, 5544-54, 2007

- *Including angiogenesis in mechanoregulation models of tissue differentiation*. Checa, S. and Prendergast, P. J. (2008) *Annals of Biomedical Engineering*. (Submitted).

6. Prospection of potential companies that could be interested in the results of the project.

7. Dissemination paper at The Parliament Magazine

8. Appearances in Spanish Media (newspapers, radio, TV)

9. Presentation of the SmartCaP project at EuroBio 2007, held in Lille (France) from 26 to 28th September