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NMP3-CT-2005-516943 – NANOBIOCOM: INTELLIGENT NANOCOMPOSITE FOR BONE TISSUE REPAIR AND REGENERATION

Executive Summary

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EXECUTIVE SUMMARY

Project Summary

There are roughly **1 million cases in the USA and ½ million in EU of high skeletal defects a year**. All of these cases require bone-graft procedures to achieve union, each of which requires the surgeon to determine the type of graft material to be use. The toughest challenge appears **when the size of the defect is too big** and the reconstruction of this defect requires a bone graft capable of supplying similar physical properties and behavior to the bone being substituted. Unfortunately at this moment commercial scaffolds can not satisfy the following issues:

- **To promote new bone formation** in order to reduce the time of bone healing and decrease the vascular insult of the implant to the bone and cause less-stress shielding.
- **Mechanical properties that match those of human tissue to be regenerated** during its new formation.
- **Large segment of implants.** For patients who have lost large segments of bone due to a congenital defect, degenerative diseases, cancer or accident.

Based on these basic needs to provide an ideal scaffold**, the NANOBIOCOM project has establishing the scientific and technological basis for the development a new "intelligent" composite scaffold for bone tissue repair and regeneration with the following issues:**

- **bioactive behavior capable of activating osteoprogenitor cells and genes and within an in vivo environment provide the interface to respond to physiological and biological changes,**
- **Mechanical and structural properties are similar to a healthy bone.**
- **Size and shape as required for reconstructing big skeletal defects**

NANOBIOCOM have developed an intelligent material with the following challenges:

- **The bioactivity of the composite**, which is rendered **by the bioactive components** (nanoparticles, carbon nanotubes, polymers) in the composite and/or **by external stimulation** (BMP's, Electrical Fields, biofunctionalitation) will active osteoprogenitor cells and genes, and consequently promote the tissue growth adjacent to the implant.
- **Mechanical and structural properties of the scaffold equal to a healthy bone** synchronous with new bone formation. By the incorporation of nanoparticles as carbon nanotubes and nanohidroxiapatite into the composite are expected to be highly suitable reinforcement for the implants of the load bearing structures of our body such as bone and cartilage.
- **The size and shape of biodegradable implants**. We are going made large segments of implants required for reconstructing big defects capable of supplying similar physical properties and behavior of healthy bone to be replaced, in contrast with the small implants that are made at present.
- **Understanding the genetic programming of bone regeneration**. To realize the potential for novel "intelligent" composite materials to serve as scaffolds for bone regeneration requires that the innate programming mechanisms involved in bone development and repair are effectively harnessed.

Project objectives

The main objective of the NANOBIOCOM project is to establish the scientific and technological basis for the development new "intelligent" composite scaffold for bone tissue repair and regeneration with bioactive behavior capable of activating osteoprogenitor cells and genes and within an in vivo environment provide the interface to respond to physiological and biological changes, with mechanical and structural properties similar to a healthy bone and with size and shape required for reconstructing big skeletal defects.

The main objectives proposed were:

- **To optimize osteoblast activation** (such as cell proliferation, collagen type I formation, and > 50% accumulation of calcium-containing mineral in the extracellular matrix) pertinent to new bone formation.
- **To achieve an "intelligent" malleable composite for 3D scaffold with match mechanical bone properties.** Mechanical properties: compressive/tensile strength, and Young's modulus close to normal cortical bone (σ_C = 25…50 MPa ; σ_T = 5…10 MPa ; E = 15…20 GPa) it should maintain while new bone formation and macroporosity Between 200-400 μm to allow for nutrient transport as well as the tailoring of polymer surface chemistry for biological recognition.
- **Genetic analysis of the processes controlling bone regeneration**. The objective is to understand the temporal and spatial organization of bone regeneration occurring in fracture repair_and to use this understanding to inform the engineering of novel scaffolding materials.
- **Increase the potential medical applications.** The main application targeted concern large lost of segments of bone in order to increase the rate of new bone formation but this knowledge could be apply to other skeletal defects (cartilages,..).

Participant List

The core group of the NANOBIOCOM consortium is composed of 8 public and private organisations coming from 5 different European countries: Spain, Italy, Switzerland, The Netherlands and U.K.

Table I: in NANOBIOCOM

Partic. Role	Partic. No.	Participant name	Participant short name	Country	Date enter project	Date exit project
CO	1	Fundación Inasmet	INASMET	E	Month 1	Month 36
CR	2	University Medical Center Nijmegen	UMC Nijmegen	N	Month 1	Month 36
CR	3	Italian Consortium on Materials Sci. & Tech	INSTM	I	Month 1	Month 36
CR	4	Instituto de Biomecánica de Valencia	IBV	E	Month 1	Month 36
CR	5	University of Aberdeen	UNIABDN	U.K.	Month 1	Month 36
CR	6	Istituti Ortopedici Rizzoli	IOR	I	Month 1	Month 36
CR	7	Ecole Polytechnique Federale de Lausanne	EPFL	SW	Month 1	Month 36
CR	8	Progenika Biopharma S.A.	Progenika	E	Month 1	Month 36

CO= Coordinator; CR= Contractor

WP1- MAIN NANOCOMPOSITE REQUIREMENTS

Leader: **IBV** Institute of Biomechanics of Valencia (Dr. Jose Luis Peris)
Partners: All partners.

NANOBIOCOM material requirements were defined in the deliverable 1.1" *User requirements report";* the characteristics of proposed materials were explained in the deliverable 1.2, "*List of possible polymers, nanoparticles and BMP's*". And deliverable 1.3 presented a list of every material that will be used by the partners in the Nanobiocom's project. A summary list of requirements is included here.

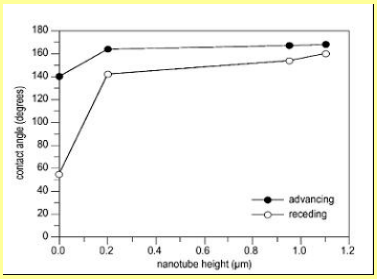
Mechanical parameters:

Parameters		Criteria	Model	Comments
Mechanical and geometrical	**Porosity**	40 < X ≤ 90%		Closely related to scaffold degradation rate
Mechanical and geometrical	**Poro size**	~ 300 μm		Direct osteogenesis
Mechanical and geometrical		< 300 μm		Endochondral bone formation
Mechanical and geometrical	**Micro- and macroporous structure**	< 10 μm		• Results in larger surface area that is believed to contribute to higher bone inducing protein adsorption as well as to ion exchange and bone-like apatite formation by dissolution and reprecipitation. • Macroporosity should be interconnected. • Interconnections larger than 30 microns
Mechanical and geometrical	**Roughness**	Ra < 2 μm and Rm > 10 μm (30 μm)	In vitro	Osteoblast with a flattened fibroblastic morphology
Mechanical and geometrical	**Strain**	5%	In vivo	Intramembranous bone formation
Mechanical and geometrical		< 15%	In vivo	Endochondral bone formation
Mechanical and geometrical		> 15%	In vivo	Connective tissue
Mechanical and geometrical		5%	In vitro	Proliferation and TGF-β increasing
Mechanical and geometrical		> 4%	In vitro	Osteoblasts turn away from the principal strain axis and avoid larger deformations
Mechanical and geometrical	**Young modulus**	50-100 MPa	FEA	To maintain strain values from 5-15%
Mechanical and geometrical	**Strength**	≥15-25 MPa	FEA	• Assuming a Young modulus of 50 Mpa. • 5% SWCNTs on different materials lead to a 150 MPa tensile strength. This strength represent a three times higher than original material strength.

Cells needs

Cell needs	**In vitro vs in vivo conditions**			Nanocomposite could respond in a different way depending on in vitro or in vivo conditions
Cell needs	**Cell migration vs cell adhesion conditions**			Directed cell migration using EFs requires adhesion of the cells to the surface, but the stronger this adhesion the less the cells will migrate
Cell needs	**Cell culture conditions**	Primary cells derived from human tissues	In vitro	They have to be preferred to osteoblast-like cell lines, as they respond to micro/nano-topography and provide 'true'mineral formation
Cell needs		Protein adsorption	In vitro	
Cell needs		Osteoblast adhesion	In vitro	It is mediated by **integrins** binding to RGD (and heparin-binding) sequences and **focal adhesions**
Cell needs		Osteoblast proliferation	In vitro	It has to be achieved **in due time**, and **entrance** of osteoblasts into pores has to be allowed by proper pore size
Cell needs		"Differentiating" culture medium	In vitro	Culture medium has to be shifted to a 'differentiating' one, and **collagen/osteocalcin/mineral** production assessed
Genetics	**Gene expression**		In vitro	Gene expression is monitored at relevant time endpoints during growth of osteoblasts onto composite
Cytokines	**Cytokine supply**	< 40ºC		Concerns related to temperature during manufacturing and/or scaffold sterilisation

Material

Material	**Material characteristics**			• Significant increase in protein adsorption and osteoblast adhesion was shown to occur on the nano-sized ceramic materials compared to traditional microsized ceramic materials; the same enhancement occurs on nanosized carbon fibers • nano-HA and B-TCP give mechanical properties to polymer and buffers low pH generated by low MW degradation products by hydrolysis of polymers • Polycaprolactone may be more desirable for the survival of thin scaffolds implanted in vivo
Material	**Biodegradation aspects**	12-15 weeks	In vivo, critical size defect	• PLA/PGA degradation affects differently in vivo or in vitro. • Currently there is no information on the degradation rate of CNTs. Until now they have not degraded in ambient conditions.
Material	**Surface wettability of CNTs**	> 150º		 Figure 1. Dynamic water contact angle measurements on carbon nanotube forests. Hysteresis, the difference between the advancing and receding angles, decreases with increasing forest height, for the same nanotube diameter and spacing.
Material	**Surface wettability of nanocomposite**	Contact angle < 30º		• It is considered as beneficial for protein adsorption, and thus for cell adhesion. • If we work with 1 %wt of CNTs, it does not depend on the SWCNTs but on the matrix and the condition processing.
Material	**Processing conditions**			
Material	**% CNT**	≈ 1% SWCNTs		• The CNTs' percentage has to be connected to the percolation threshold of the electrical properties and thereafter check the variations in the mechanical properties and tune these. • The CNTs' percentage is connected to the tube dispersion in the matrix.
Material	**SW-CNT vs MW-CNT**	SWCNTs		• Carbon nanotubes are used to improve the bioactivity of the composite by means of specific biofunctionalization and to increase the mechanical properties of the matrix. Therefore, it is very important to optimize the process of carbon nanotube functionalization. • In the case of SWCNTs, it is more simple to control the functionalization of the system because there is only one wall that interacts with the functional groups. Whereas, in MWCNTs, during the biofunctionalization process, it is difficult to understand the physical-chemical interaction of the different walls.
Material	**Electric functionalisation conditions**	< 1% SWCNTs		Related to CNTs: • non covalent functionalization between the matrix and SWNTs, in order not to modify the good electrical properties of the nanotube; • to use very low percentage of CNTs in the composite (<1% wt); • electrical properties depend especially on the concentration of CNTs in the polymer and not on the matrix; • there is a percolation threshold; • conductivity can increase 10 order of magnitudine with 0.8 %wt of CNTs in an epoxy matrix; • conductivity could increase using an alignment process during composite formation, applying an electrical field.
Material	**Biocompatibilty**			Different results and very limited toxicology information about the effect of CNTs on biomaterials.
Material	**Calcium phosphate**	Low levels		Optimum cell migration
Material		High levels		Stimulation of phenotype expression

WP2- PRODUCTION AND CHARACTERIZATION OF NANOCOMPOSITE

Leader: Ecole Polytechnique Fédérale de Lausanne -EPFL (Prof. Jacques Lemaitre)
Partners:

- **EPFL:** Ecole Polytechnique Fédérale de Lausanne (Prof. Jacques Lemaitre)
- **INASMET** Fundación Inasmet-Tecnalia (MsC Mª Jesus Jurado)
- **IBV** Institute of Biomechanics of Valencia(Dr. Jose Luis Peris)
- **INSTM** University of Perugia (Prof. Jose Kenny)

Objectives
The original objective was to develop an « intelligent » bone substitute material matching the following mechanical healthy bone properties:

- Intrinsic mechanical properties (dense material) close to those of cortical healthy bone : σ_C = 180…215 MPa, E_C = 25…35 GPa ; σ_T = 145…170 MPa, E_T = 19…30 Gpa
- Apparent mechanical properties (macroporous material) close to those of healthy cancellous bone : σ_C = 5…10 MPa, E_C = 15…20 GPa ; σ_T = 145…170 MPa
- Material incorporating an interconnected macroporous network, with a minimum pore diameter of 200…400 micrometers in order to enable early vascularization and cell penetration in the bulk of the material
- Bioresorbable material, preferably through a process close to bone remodelling
- Osteoconductive material, *i. e.* allowing new bone growth in close apposition to the residual material.

However, after establishing the main nanocomposites requirements for bone substitutes in WP1, the subsequent WP2 addressed to the following **hypothesis**:

0. Mechanical properties of the scaffolds: Young modulus. 50-100MPa; Maximum stress (strength) 15-25 MPa and Strain: 5-15%.
1. Porosity of the scaffold: Total porosity: 40%>X>90%; Microporosity: ≤10µm; Macroporosity: > 300 µm
2. Bioresorbable material, preferably through a process close to bone remodelling (18months)
3. Osteoconductive material, *i. e.* allowing new bone growth in close apposition to the residual material. (to be proven after WP5)
4. Electrically conductive scaffold (to be proven after WP3.2)

For completing phases WP3.3 tasks, it was necessary to start culturing the cells on composite material, as devised in WP2. A large batch of porous composite scaffold material, made of poly lactic acid (PLLA), carbon nanotubes (CNT) and nanohydroxyapatite (nHA), was prepared for this task. All necessary protocols etc. were completed, and cell studies were initiated. However, cell culture proved more time consuming, as no cells would adhere to, or proliferate on the porous scaffold composite so far. A cytotoxicity test using dermal fibroblasts showed that the material is not compatible with cell culture so far. Ongoing investigations in a fruitful interaction with the material scientists working in WP2, have proven these problems have arisen from a decreasing pH and dissolution/precipitation on the surface of the composite, when it is submerged in cell culture medium. This decrease in pH was noted when the composites were placed into the cell culture medium, which is used in low volume to obtain a high enough cell density for optimal loading of the cells.

Two studies were carried out to understand these findings. The first one was the Acidification of the culture medium. It was concluded that only nano size HAp has a strong influence in the pH and ions content.

A second study was developed to find Alternative Ca sources for the nanocomposite. It was observed that higher quantity of percentage of HAp (nano and micro size) was used the pH and the ion content of the culture medium decrease stronger. Despite these facts, no cytotoxtic effect was observed.

Taken into account these conclusions, the final agreement for the Scaffold optimization step were to produce scaffold with 1% of µHAp with the same TIPS process conditions **(**Solvent: Dioxane/Water: 87/1, Tquenching: -16ºC and Composition: 10% PLLA (w/v), 1% SWNT (w_{cnt}/ w_{PLLA}) and 1% µHAp($w_{\mu HAp}$/w_{PLLA}).

Final answer to our initial hypothesis:

0. Materials fulfil Young modulus criterion (> 50 MPa) but non of them achieve maximum stress criterion (>15 MPa)
1. Porosity of the scaffold. 70-90%; Microporosity: ≤10µm; Macroporosity: > 300 µm
2. Bioresorbable. OK
3. Osteoconductive materials. In vivo experiments showed that nanobiocom materials support bone growth and encourage the ingrowth of surrounding bone
4. Electrically conductive scaffold. A conductive nanocomposite was obtained with 1 percentage in weight of CNTs.

WP3- SPECIFIC CELLS ACTIVATION

Leader : UMC Nijmegen (Dr.XF Walboomers)
Partners :

- ***UMC*** : Radboud University Nijmegen, the Netherlands (Dr. XF Walboomers)
- ***IOR*** : Istituti Ortopedici Rizzoli (Dr. G Ciapetti)
- ***PROGENIKA*** : Progenika Biopharma S.A. (Dr G Ochoa, Dr L. Osaba)
- ***ABERDEEN:*** University of Aberdeen (Dr. I Gibson)
- ***INSTM*** : Italian Consortium for Materials Science and Technology (Dr. JM Kenny)

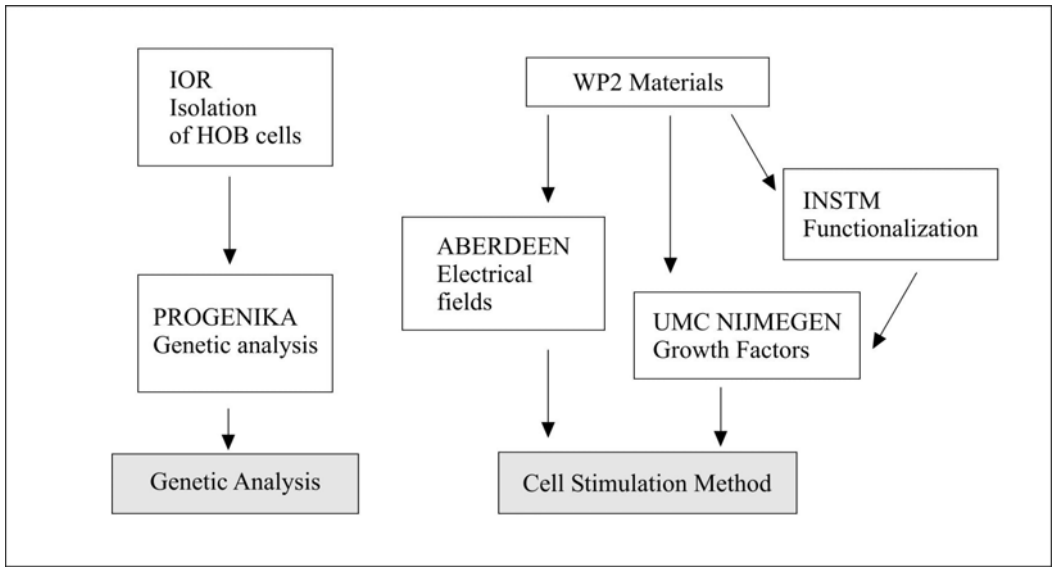
Objectives
This workpackage is the core of project and its main objectives is to optimize osteoblast activation (such as cell proliferation, collagen type I formation, and accumulation > 50% of calcium-containing mineral in the extracellular matrix) pertinent to new bone formation. The WP3 can be considered as the main working line as it is dedicated to SPECIFIC CELLS ACTIVATION. WP3 will be divided in four sub-workpackages:

WP 3.1- GENETIC ANALYSIS OF THE PROCESSES CONTROLLING
WP 3.2- ELECTRICAL STIMULATION
WP 3.3- MORPHOGENETIC FACTORS
WP 3.4- BIOFUNCTIONALITATION

Hypotheses
After production of a NANOBIOCOM material (Polymer matrix, with a dispersed phase of both hydroxyapatite nanocrystals and carbon nanotubes) in WP2, the subsequent WP3 will address to the following hypotheses:

1. A fully genetic analysis of the human osteoblasts is feasible (hypothesis to be proven after WP 3.1)
2. Electrical stimulation will be a more potent stimulus for adherent bone cells than growth factor addition (to be proven after WP 3.2 / 3.3; cannot be true unless #3 is false)
3. Growth factor addition will be a more potent stimulus for adherent bone cells than electrical stimulation (to be proven after WP 3.2 / 3.3; cannot be true unless #2 is false)
4. Biofunctionalization of the polymer will have a synergistic positive influence on adherent bone cells (to be proven after WP 3.3 / 3.4)

Research program
The research steps are depicted in the diagram below:



Final answer to our initial hypotheses

1. True; a fully genetic analysis of the human osteoblasts was provided
2. False; BMP stimulation is preferred over electric field stimulation
3. True; the growth factor BMP-2 is the most powerful stimulus for bone cell development
4. True; biofunctionalization has a positive influence on adherent bone cells

WP3.1- GENE ANALYSIS RELATED TO BONE CELL ACTIVITY

The 233 genes selected and the validation results have been recorded in a database which includes for each gene the following information:

- Gene title
- Link to the 'EntrezGene' database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene)
- Gene symbol
- Differentiation or mineralization step(s) in which the differential expression was found
- Fold change, or fold-change range
- 'Gene ontology' annotation of biological process(es) in which it is involved (http://www.geneontology.org/)
- Link to GENMAPP (http://www.genmapp.org/) and/or KEGG pathway (http://www.genome.jp/kegg/pathway.html)
- Annotation about the biological function retrieved from PUBMED (only for the 123 genes not included in any specific biological pathway)
- Real Time PCR results: data with fold change > 3 and/or p value < 0.05, and data with 2 < fold change < 3 and p value < 0.1 have been accepted as, while data with 2 < fold change < 3 or p value < 0.1 have been classified as not conclusive (doubtful results).
- The database is enclosed as supplementary file (hyperlink: NANOBIOCOM_List of selected genes_validation.xls).

Summary of the achievements:

An experimental protocol specifically designed to mark the crucial steps of differentiation and mineralization of bone forming cells in vitro has been devised. The scaling-up of the

experimental draft described in the initial proposal (2 types of bone-forming cells vs 1, four patients vs 3, multiple timepoints) is bound to add significance to the results obtained.

The final list of selected genes represents a useful tool to understand the precise temporal gene expression patterns related to bone cell differentiation when bone cells are cultured onto scaffolds. The link to external databases allows a continuous up-grading of the information related to genes of interest.

WP3.2- ELECTRICAL STIMULATION

Overview

The objectives of the work-package (WP 3.2) at the University of Aberdeen are to determine how the migratory and phenotypic responses of osteoblast-like cells are affected when grown on novel polymeric-carbon nanotubes (CNT) composites, in the presence and absence of applied electric fields effect (EFs).

Summary of the achievements:

The initial goal was to evaluate if cell behaviour could be guided/stimulated by combinations of conducting nanocomposites and applied d.c. electric fields (EFs). These results suggest that applying an EF enhances the differentiation of osteoblast cells. There was no significant difference between the PLLA and PLLA/CNT surfaces, suggesting that the presence of CNT did not affect cell phenotype, but notably did not inhibit proliferation or differentiation in contrast to the results observed for cell migration. The goals of this study have been achieved, where certain combinations of sample composition, applied EF, and EF configuration can direct osteoblast behaviour. These different combinations may be potentially applied to different applications/technologies (e.g. enhanced healing of implants, accelerated migration of cells into tissue-engineered scaffolds in vitro, electric field therapies for wound healing).

One of the most important stages in wound healing/bone repair is the formation of a new blood supply, and this is governed by the migration of endothelial cells. The goal of this study was to determine if we could use the composites and/or applied EFs to direct human endothelial cells (human vascular endothelial cells, HUVEC). In a similar way to the osteoblast studies, we found that the migration of HUVEC cells grown on PLLA could also be directed towards the cathode when an EF was applied through the culture medium; PLLA/CNT composites did not allow directed cell migration. However, PLLA/CNT composites could ‘attract’ HUVEC cells when it was used as the cathode and an EF was applied, causing HUVEC cells attached to tissue culture plastic to migrate towards the PLLA/CNT composites.

These studies provided evidence that combinations of applied electric fields and conducting nanocomposites can be used to direct the migration of endothelial cells. As result of this, an industrial patent is done.

WP3.3- MORPHOGENETIC FACTORS

Bone inductive proteins can be used to induce bone growth when loaded onto composite materials. In the WP3.3 different bone inductive proteins were tested for further use in the remaining part of the project. The remaining part consists of examining the release and bio-activity profiles of this growth factor on the composites in vitro, and in vivo. Next to that, an extra task was performed; i.e. biodistribution of nanotubes, after composite degradation, was visualized using gadolinium labelling and MRI detection.

From the loading/release experiments it can be concluded that:

- BMP2 is released in a burst release within the first 4 hours, after the first hours BMP-2 is released gradually up to 40-50% of the total amount.
- BMP2 remains bioactive after release.

From the DNA/ALP and SEM analysis experiments the following conclusions can be made:

- BMP-2 seems to have a negative effect on proliferation and a positive effect on differentiation.
- The added CNT and mHA seems to have a negative effect on both the proliferation and differentiation of the cells, the negative effect on differentiation is especially significant.

From the gene profiling experiments the following conclusions can be made:

- The effect of BMP-2 without CNTs results in a downregulation of genes in the apoptotic and cell proliferation processes, and has a positive effect on skeletal development.
- The effect of CNTs in combination with BMP-2 results in an upregulation of genes involved in apoptosis, cell proliferation and vasculature development, and in a downregulation of skeletal development.
- CNTs alone do not seem to have an adverse effect on bone development, in contrast to the combination with BMP-2.

The growth factor BMP-2 can be loaded onto, and is released from the NANOBIOCOM scaffold successfully. BMP after release stays present in an area around the material, for up to 3 weeks until all is growth factor cleared from the region.

Central in Nanobiocom and WP 3.3 are the biological characteristics of a new scaffold material composed of PLLA/µHA/CNT. Literature describes the positive effects of carbon nanotubes in scaffold materials i.e. increasing mechanical strength, the possibility to functionalize CNTs, for instance with growth factors to acquire a bioactive scaffold, and increasing osteoblast proliferation [1]. However, little is known about the distribution of these CNTs after degradation of the scaffold. Some articles even report on toxicity of CNTs while others do not describe such effects. Intratracheal instillation of the SWNTs showed a toxic effect with severe inflammatory responses; however this might have been due to agglomeration of the tubes [2]. On the other hand, subcutaneous implantation of carbon nanofibers did not show severe inflammatory responses [3].
The promising characteristics of this material have raised the need for a deeper understanding of the toxicological properties. To start with the questions; if CNTs are used in a degradable scaffold, how are they distributed through the body after release? Are they cleared from the system or do they accumulate in certain organs, and if they do, are they toxic? Therefore we designed this additional task to investigate the distribution of single walled carbon nanotubes after in vivo degradation of a scaffold, using Magnetic Resonance Imaging (MRI).

- Gadonanotubes can be visualized in vivo with MRI and scaffolds are visible as black disks, due to the impermeability of water
- After release, the gadonanotubes seem to be located in the surrounding tissue with a peak around day 18 and are systemically cleared afterwards, or at least present below the detection limit.

BMP-2 was chosen for future experiments to improve proliferation and differentiation of osteoblast-like cells on nanocomposites.

WP3.4- BIOFUNCTIONALIZATION

The main objective of this study is the improvement of polymer biofunctionality and scaffold ability to interact with the cells that can be obtained increasing the nanotube-polymer and nanotube-cell affinity.

Biofunctionalization was investigated in different ways in the four tasks:

- Surface modifications of the nanocomposite

PECVD is a suitable technique to modify surface properties of nanocomposite based on CNTs and polyester polymer. It was demonstrated that plasma treatment does not modify the good electrical properties of CNT nanocomposites. It was shown that this kind of treatment is suitable for porous material.

Oxygen plasma was shown to uniformly activate the surface of PLLA without affect the bulk properties. The effect of the plasma treatment on the surface of based material is to create hydroxyl groups that improve the selective interaction with the protein (PLLA-BSA). The experiments and measurements showed that Oxygen and CF_4 plasma treatment increase the cell affinity.

- SWNT-functionalization.

Functionalized SWNTs were performed. Fluorinated and carboxylate SWNTs were characterized. In particular investigations using human marrow stromal cells show that these cells are allowed to attach and grow onto thin layers of SWNT-COOH. As far as the cell compatibility of functionalised carbon nanotubes is concerned, the SWNTs-COOH films allow for human marrow stromal cell adhesion and proliferation.

- Polymer-SWNTs interaction

F-SWNTs and SWNTs-COOH showed good interaction to the polymer matrix. It has been observed that system composition and SWNT functionalization may play a crucial role on the autocatalytic effect of the degradation process. In particular carboxylate tubes revealed a better grade of dispersion in solvent and matrix and an increase in mechanical properties and for this reason they are suitable for nanocomposite development.

WP4- INTEGRATION AND MATRIX VALIDATIONFINAL REPORT

Objectives

- Integration of cells activation WP's results.
- Isolation, expansion and characterization in vitro of human primary bone-forming cells.
- Seeding of the 3D composites and analysis of the bone-forming activities on the constructs Mechanical and histomorphometrical evaluation of the 3D cultures.
- Complete in vitro testing of bone-forming cell/composite construct.

Workpackage 4 represents a critical task of the project, as it starts from the integration of the results collected in WP3 about the efficient methods for cell activation and goes through the preparation of samples of the selected nanocomposite up to the evaluation of the bone-forming activity of the 'intelligent' composite following cell seeding and extended *in vitro* culture.

The different PLLA-based composites developed by the Nanobiocom consortium were deeply analysed in vitro after seeding of rabbit and human marrow stromal cells and extended culture.

Potential activation systems include the application of electrical fields, the presence of BMP2, the addition of 1% single wall carbon nanotubes (SWNT) and 1% micro-hydroxyapatite, and the treatment of the surface with plasma technique.

Main achievements

The achievements on the activation systems could be summarized. The best results in terms of bone formation activities *in vitro,* as recorded using morphological, biochemical and molecular assays are obtained with the loading of BMP2 on the CNT-HA-PLLA scaffold, which is able to support proliferation and differentiation of primary marrow stromal cells more than any other scaffold assayed.

WP5- PRECLINICAL TEST

Objectives

- To establish an experimental model of fracture healing and non-union by means of a critical size long bone defect.
- To perform experimental surgery in animals reproducing the bioactivity and non-union model.
- To implant the matrix 3D culture and assure his restoration capability
- To evaluate bone quality at the fracture site of different experimental groups by means of several analysis.
- Assure the viability of nanocomposite as medical device.
- Assure the safety of use of potential future industrial application

Main achievements:

Experimental design of animal model. Unilateral 15-mm mid-diaphyseal critical-sized defects were created in the radii of sixty-six skeletally mature New Zealand White rabbits. The defect was validated as being of critical size on the basis of data from a previously reported study of the same model. Skeletal maturity was verified by the radiographic appearance of closure of the physeal plates. The defects in twenty-four rabbits each were treated with autograft, in other twelve animals the defects were treated with the NANOBIOCOM scaffold with rhBMP-2 and, finally the defects of the other twenty-four animals were treated with the NANOBIOCOM scaffold without rhBMP-2. In the sixteen-week groups only NANOBIOCOM scaffold with rhBMP-2 and autograft were used. Group sizes were determined on the basis of a power analysis done in the previously reported studies.

Histology, immunochemical and microscopical examination were carried to evaluate the bone formation and material. NANOBIOCOM scaffold (with or without rhBMP-2) showed no signs of an adverse inflammatory reaction. High density bone tissue has grown around the implant and bone remodelling process has begun allowing radius formation.

Gene Expression was studied in fractured zones of rabbit bone. Mechanical analysis of healing was also studied with Four-point bending test and torsional tests simulate clinically relevant fracture patterns. **Morphology**-Three-dimensional analysis was conducted on all implanted radii and on all intact contralateral ones.

To assure the viability of nanocomposite as medical device. The nanocomposite developed in this project is intended to be a **BONE SUBSTITUTE**, so by ISO 10993-1 recommendations it should be categorize as an **IMPLANT DEVICE** to be in a **PERMANENT CONTACT** (C: more than 30 days) with BONE.

The following tests were carried out:
Citotoxicity ISO 10993-5. It can be concluded that the tested extracts of the samples are no-cytotoxic.

Genotoxicity ISO 10993-3. Bacterial reverse mutation. The tested concentrations of the sample did not result significant mutagenic and did not affect the bacterial survivals, so the sample can be considered "no mutagenic".
Irritation ISO 10993-10. The material tested does not cause irritation response.
*Sensibilization ISO 10993-10. Guinea Pig Maximization test (GPMT)***.** The material tested in this project does not cause sensitization response.

Conclusions

- At 16 weeks we obtain with NANOBIOCOM material similar values to autograft and slightly lower than intact bone. No statistically significant differences.
- NANOBIOCOM scaffolds with or without rhBMP-2 were able to regenerate bone in rabbit radius with comparable mechanical strength to autograft-filled defects.
- From the point of view of biocompatibility and according to the results of the realized tests, the nanocomposite developed in this project is a good candidate as a Bone substitute. None of the biological test performed during the Project have indicate any toxicity.

General conclusions

- **We have obtained a bioactive composite.** This property was rendered **by the bioactive components** (microHidroxiapatite, carbon nanotubes, Polylactic polymer) in the composite and **by external stimulation** (BMP-2). The composite was able to active osteoprogenitor cells and genes, and consequently promote the tissue growth adjacent to the implant until new bone formation.

- **Mechanical and structural properties of the scaffold are equal or higher to a healthy bone** synchronous with new bone formation. The incorporation of particles such as carbon nanotubes and microhidroxiapatite into the composite are suitable reinforcements without decreasing of mechanical properties.

- **The size and shape of biodegradable implants**. We made large segments (>15 mm length, 4 mm Ø in rabbit) of implant required for reconstructing big defects with similar physical properties and behavior of healthy bone to be replaced, in contrast with the small implants that are made at present.

- **We are able to understand the genetic programming of bone regeneration**. We have developed a tool to analyze the potential for any novel "intelligent" composite materials to serve as scaffolds for bone regeneration. That means, innate programming mechanisms involved in bone development and repair are effectively understood and may be applied.

The main outputs of NANOBIOCOM project, from the biomedical point of view, are:

- **An appropriate cell bioactive system** to initiate repair and regeneration of bone.
- **Intelligent composite for 3D scaffold**, which is able to support physiological loading until sufficient tissue regeneration occurs and will be possible manufacture large segments of material.
- **A comprehensive gene expression profiles** of the temporal regulation of genes during bone development were obtained "in vitro" (human, rabbit and mouse) even "in vivo" experiments.

In addition,

- **Long time cell culture is possible with this type of materials**. The control of the material properties developed during the project let obtain tailored microstructures able to support long time cell cultures and act as cellular scaffolds.

- **No sign of toxicity, induced by the composite material has been observed.** These promising results encourage further developments of this technology for clinical applications.