2011

P1

During **activity 2.6** we have further developed the results obtained in the first stage by conducting studies of celuular senescence on ADAS cell cultures and immunophenotyping (flow cytometry). These studies have brought important data necessary to carry out properly the 3-D studies.

In the **activity 2.11** it was revealed the ADAS cells' MMP2 profile by gelatin-zymography. The results indicate an increase in the enzymatic activity of MMP2 during the 21 days of adipogenic induction. A first step to assess biocompatibility and cell differentiation in the systems proposed by the partners P3 and P7 was the optimization of 3-D seeding.

Activity 2.3 was the most laborious due to the large number of samples and the variability of test methods and protocols. Biocompatibility studies performed by P1 team were:
i) assessmet of the scaffolds cytotoxic potential on the embedded cells
ii) evaluation of the cellular viability and proliferation through: MTT assay (spectrophotometric quantification of the concentration of formazan formed by metabolically active cells) and fluorescent labeling (fluorescence microscopy and flow cytometry)
iii) evaluation of cell-support system morphology by SEM.

The **activity 2.8** was performed using the 3-D structures previously selected. So far studies have been completed on two of the four proposed systems (Coll-H / Coll-Ser-H and RH / CGH). Adipogenic differentiation was assessed by highlighting inracytoplasmatic lipid accumulation by Oil Red O staining, and by studying the expression of adipogenic markers such as: PPARγ2, aP2 and perilipină by RT-PCR and / or flow cytometry.

P2

P3

In this phase **Partner P3 – University Polytehnica of Bucharest – Faculty of Applied Chemistry and Materials Science** has made several series of polymeric substrates in the form of swollen hydrogels (2D systems) or 3D porous networks from various natural (collagen, sericin, gelatin, alginate, glycosaminoglycans) or synthetic (polyacrylamide) components. Various polymeric scaffolds with suitable features for the project objectives for 2011 were selected by P3 from all the obtained samples.

Thus, in order to accomplish the **objective 1** of the project (activities **1.1, 1.3, 1.5**), various hydrogels consisting in two protein-based polymers: ***collagen (constant content) and variable concentrations of silk sericin*** **were developed by P3 and supplied to P2** in order to establish their biological features. Physicochemical, thermal, morphological and mechanical features proved that this scaffolds are suitable for bone tissue engineering. Hydroxyapatite loading was tested also in order to obtain scaffolds with an increased resistance.

Within **objective 2**, P3 has been involved in various activities **(2.1, 2.4, 2.5, 2.9**). Binary and ternary hydrogels were obtained and characterized by P3 and subsequently **provided to P1** for biological testing. Firstly different experimental compositions based on gelatine-alginate

were tested and after a synthetic component was added (polyacrylamide). The porous scaffolds based on ***gelatin-alginate-polyacrylamide*** were obtained in the form of interpenetrating networks through a step-by-step procedure starting with the polymerization of acrylamide and followed by the crosslinking of the natural components. Also, 3D scaffolds based on ***collagen and variable concentrations of silk sericin*** were obtained by P3. The resulting materials both as swollen hydrogels or 3D porous scaffolds were advanced investigated to evaluate their physicochemical (FTIR, UV-VIS, XPS), thermal (DSC si TGA/DTG), morphological (SEM) and mechanical features.

The main aspects of interest in the SEM micrographs were the homogeneity of the micro-architecture, the orientation, the interconnectivity, the size and shape of the pores. The morphology of the porous scaffold is very important because it affects a series of features like water uptake capacity and the moisture absorption as well as the mechanical properties. The geometry and the interconnection of the pores strongly influence the water transport efficiency within the porous scaffolds and the diffusion of nutrients into the scaffold as well as the colonization of the materials with cells and blood vessels. The obtained materials could be used as scaffolds for different cell types depending on the initial ratio between the raw materials due to the fact that all these properties can be modulated by slightly change the initial polymer mixtures.

P4

Main activities for P4 in phase II are related to the theme

1.4. Processing metallic alloys in order to increase their biocompatibility

e) Surface analysis (AFM, SEM, contact angle) and elecrochemical characterization The nanotubes were elaborated A) in mixtures $(NH_4)_2SO_4$ 1 M + NH_4F 0.5 wt% (electrolyte1) and B) in glycerol + 4 % H_2O + NH_4F 0.36 wt %, (electrolyte2). Elemental analysis indicated different composition as a function of electrolyte. Using contact mode method for AFM roughness was determinated and computed with Gwyddion program. The contact angle determinated the hydrophylic-hydrophobic balance at surface. The Ti and Al ion release from Hank bioliquid at 37ºC was evaluated from ICP-MS for maximum 46 days. The obtained corrosion rates have values close to corrosion rates from Tafel plots.

f) Structural characterization (FTIR) and surface analysis (AFM, SEM, contact angle) for electrodeposition of polymer composites obtained on Ti alloys (TiAlNb si TiAlZr). The Ppy structure obtained in aqueous solution 0.2 M oxalic acid contains small grains and the film is more compact comparing with the film fabricated in aqueous solution 0.1 M $LiClO_4$, where the grains have higher dimensions and the roughness is higher. Regarding infrared spectra in all electrodeposition the caracteristic bands of polymers were present.

g) The electrochemical stability and ion release from modified electrodes with polymer coatings in simulated bioliquids, (TiAlNb and TiAlZr alloys). The amount of Ti and Al from Ti6Al7Nb untreated and coated with polymeric films in Hank bioliquid at 37ºC indicated a much smaller value for Al ions release. In the case of coated with polymer Ti6Al7Nb alloy the amount of Ti and Al ion release is increasing due to the dissolution of polymer stratum and release of corrosion products. After 14 immersion days the amount of ion release is constant

until 46 days. A range of polymer film stability is: PPy/PEG > PPy/NaPSS (NaPSS is a surfactant).
The behavior of polymeric film on TiAlZr is quasisimilar. The increase of film stability at surface of processed alloy is not an assurance for a better oseointegration for non apperance of the infection. Such fact in the condition of antibiotics effect decrease (due to their intensive use) leads to a need to introduce particles with antibacterial effect. (Ag Nanoparticles).
The surface antibacterial effects of nAg -TiAlZr were evaluated after substrate was exposed at Ecoli bacteria. Regarding anticorosive properties of the stratum with nAg, we can say thhat the properties are much better. In the presence of nanoparticles with specific dimensions evaluated with DLS. **Conclusion The characterization of the alloys stratum after processing lead to the increase of bioperformance quantified in better stability and antibacterial effect.**
h) The aquisition of an equipment for determination of surface charge was started and finished in this phase, the equipment being now in function.
Dissemination. This activity was much developed (4 papers ISI with cumulate impact factor of 6.71 and high influence score) comparing to prognosys (cumulated factor 5). Results dissemination at conferences permitted team members, especiialy young PhD students and postdocs, to present project results and to actively participate to increase the research visibility

P5

1.5.*c*. Establishment by electrochemical methods of the medium term behaviour of the films obtained on supports of Ti and TiAlV

Cyclic voltammetry curves of the films obtained on supports of Ti and TiAlV reveal a passiv metal behaviour that becomes nobler in time.

Nyquist impedance spectra evince incomplete semicircles with very big curvature radii that show a capacitive behaviour, a very resistant, protective layer.

1.5.*f*. Medium term morphological characterisation of the activated surfaces on supports of Ti and TiAlV by SEM

SEM study reveals the presence of a thick coating on the surface of the supports of Ti and TiAlV after 500 immersion hours in physiological solutions.

1.5.*h*. Obtaining of the passive activated films on supports of Ti and TiAlV by chemical methods

Elemental EDX analysis confirmed the formation of hydroxiapatite by the presence of calcium and phosphorus ions; low quantities of sodium ions and constituent elements were detected.

1.5.*i*. Short term behaviour by electrochemical methods of the obtained films on support of TiNbZrTa alloy

Cyclic voltammetry curves of the passive activated films characterise a stable passive state with more electropositive corrosion and passivation potentials and lower passive currents than those of the bare alloy due to the beneficial effect of the coating chemically deposited.

1.5.*k.* Short term morphological characterisation of the surfaces activated on support of TiNbZrTa by SEM

SEM micrographs obtained after 48 immersion hours of the activated surfaces in physiological solutions show a globular morphology formed by acicular crystals, covering whole support surface.

P6

Nanostructured glass ceramics were obtained by 500 ^{o}C calcination of lime phosphosilicate and soda lime phosphosilicate bioglasses. The first one is structured as hydroxyapatite, while in thestructure of soda containing sample prevails the calcite. Infrared analysis shows the presence of hydroxyapatite in both systems. The distribution of Q^n species indicates that the connectivity of [SiO_4] units is enhanced by soda addition, what could diminish the bioactivity. The cellular response tested *in vitro* proved that both compositions sustain osteosarcoma cell growth.
Aluminosilicate / polyvinyl alcohol composites of similar composition were prepared by both solution intercalation and mechanical mixing. DTA/TGA analysis points out a better thermal stability of the clay/polymer composites compared to the precursor polymer, as well as similar thermal behavior of the two composites, and implicitly a structural resemblance of the composite obtained by mixing/milling process under described conditions, and the composite prepared by solution intercalation. X-ray diffraction recordings and TEM images suggest a phase separated microstructure of both composites with a slight tendency of intercalation in the sample prepared by solution intercalation technique.

P7

The obligations as a subcontractor on the project 248/2008 had as main objective the synthesis of hydrogels used as carriers for immobilization, growth and proliferation of stem cells from adipose tissue.

The primary and eliminatory criteria for the quality of the support hydrogels was their interaction with cells. It was considered that the support is good if it promotes the growth and multiplication of cells or the contrary is inadequate if it has a harmful action on them.

Hydrogels were characterized by estimating the chemical structure (FTIR, NMR) and morphological (ESEM microscopy, gravimetry for homogeneity) and by measuring the transport properties (gravimetric), dynamo-mechanical properties during the formation of hydrogel and then after formation (DMA measurements), the thermal stability (DSC, ATD). We mention that the surface study is not representative for the 3D cellular structures. The value of elasticity modulus (E) is, according to the *theory of elastomers elasticity*, directly correlated with cross-linking density (ν) according to the relationship: $E = 3\nu RT$. If the elasticity modulus provides information on the density of the polymer network from which the hydrogels consists, electron microscopy reveals its morphology.

In the first instance it was decided, in agreement with the project manager, that P7 will study alginate-based hydrogels made by diffusion, heat-sensitive hydrogels and hydrogels cross-linked with genipin.

Alginate, linear polysaccharide with block-copolymer structure, consisting of a sequence of blocks of β-D-mannuronic acid and α-L-glucuronic gelifies in the presence of Ca salts that interacts with carboxyl groups of the gluconiric acid forming ionic bridges. The reaction is very fast and occurs in an uncontrolled manner upon contact of the two reactant solutions and, therefore, cannot be used as such, to obtain support hydrogels with controlled morphology.

The study of alginate-based hydrogels took into account the introduction of Ca^{+2} ions in the system through a physical slow process (diffusion) in order to realize the hydrogel and analyze the effects on morphology and properties of the method for hydrogel synthesis.

The method of controlled introduction of Ca^{+2} ions in the system via a slow diffusion process, proved to be appropriate both in terms of reaction kinetics as well as in terms of the interaction hydrogel - stem cells from adipose tissue. Effectuated works have shown that results depend very much on the Ca salt used. Thus the elasticity modulus of (crosslinking density respectively) increased over time, significantly faster when dealing with the crosslinking is realized with Ca gluconate instead of Ca chloride. The final value of the modulus is also, appreciably higher for gluconate. In both cases, freeze-dried hydrogels have the same type of "eggshell" morphology. The size of the cavities of the hydrogels obtained with gluconate, however, is hundreds of times higher than those obtained with chloride. At the same time cells grow and develop much better in hydrogels - gluconate than in hydrogels - chloride. If we assume that the cells are fixed in the cavities, it results that cavity size is a very important factor in cell development.

To realize thermosensitive hydrogels chitosan was used. Chitosan is a linear polysaccharide, which contains units of glucosamine and N-acetylglucosamine similar to natural glycosaminoglycans. Unmodified chitosan can be dissolved only in acid solutions due intermolecular hydrogen bonds. The solubility of chitosan in aqueous solution is achieved by the protonation of its amino groups with acid from medium. Neutralization of chitosan aqueous solution to a pH greater than 6.2 systematically leads to the formation of a gel- looking precipitate.

Chitosan solution at neutral pH can be realized by adding a dibasic polyol complex like β-glycerol-phosphate disodium. It was shown that chitosan solutions neutralized with β glycerol phosphate forms a gelifier that remains liquid for long periods at room temperature and which becomes a macroporous gel when the temperature reaches 37 °C. The combination of chitosan-β-glycerol-phosphate disodium benefits of synergetic effects favorable to gel formation such as hydrogen bonds, electrostatic interactions and hydrophobic interactions. The unique character of this combination is overcoming the barrier of pH for chitosan solution, the barrier that was long time a major limitation for many applications. Gel formation mechanism is not known in detail.

Experiences for thermosensitive hydrogels chitosan - β glycerophosphate revealed that cells do not grow on the support and dies within a few days, when β glycerophosphate concentration is greater than 1 g / 10 mm 1.5% sol. Below this concentration, the stem cells from adipose tissue have a normal behavior. Chitosan thermosensitive gels are obtained at neutral pH, the excess acid necessary for chitosan solubilization is neutralized β glycerophosphate. When in the system is introduced less β glycerophosphate, at a level that does not affect cell growth, the pH of the medium remains acid. The removal of excess acid is performed through dialysis.

To avoid dialysis, a laborious and time consuming operation, another solution was chosen, namely the dissolution of chitosan in the minimum necessary amount of acid.

Genipin is an excellent natural cross-linking agent for collagen, gelatin and other proteins as well as for chitosan. It is insoluble in water and much less toxic than glutaraldehyde and other cross-linking synthetic agents. Crosslinking mechanism is unclear until now. Chitosan-based hydrogels ionically cross-linked with β glycerophosphate and chemically with genipin were studied. The realized experiences have confirmed that genipin is active in very low concentrations and the crosslinking is achieved by chemical bonds. By comparison with thermosensitive gels, the formation speed of genipin cross-linked gels is significantly lower. Cytotoxicity tests have imposed, as a mandatory step, the washing of hydrogel before cell insertion.