Morphological Biodegradation and the Citotoxicity Effect of Some Experimental Biomaterials

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The purpose of this work was the detailed study of morphological biodegradation and the citotoxicity effect of some experimental biomaterials. The study has four experimental biomaterials based on polylactic acid, polyethylene glycol, tricalcium phosphate, chitosan and hydroxyapatite in different percent cured in air followed by heat treatment at 120^{0}C. The experimental biomaterials were characterized by AFM (Multimode V-SPM, Veeco) and SEM (Q130 -FEI Company) microscopy. For the citotoxicity were used cell cultures of normal human fibroblasts and the viability testing was made with trypan blue. According to the experimental results, the biomaterial with highest percent in polylactic acid presents a high degradation, compared with the rest of the biomaterials which have HA in composition, who decrease this process. Fibroblasts cell culture showed good adhesion to the surface of the biomaterials. The viability of the biomaterials increases at 48h compared with 24h, which suggest the absence of the materials cytotoxicity. Our results sustain idea that biomaterials based on chitosan with additions can be successfully used for further studies in reparative medicine.

Keywords: chitosan, biodegradable polymers, hydroxyapatite, SEM, AFM, citotoxicity

In the studies of biomaterials based on polymeric composition for tissue engineering, one of the main objectives is to observe their morphological behavior after natural degradation of the polymeric component [1-3].

The multitude of biodegradable synthetic polymers used for tissue engineering applications are reported on the many papers by using polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers poly (DL-lactic-co-glycolic acid) (PLGA) which are approved by the Drug Administration (FDA) [4-7]. These polymers degrade by hydrolytic mechanisms and are commonly used because their degradation products may be removed from the body as carbon dioxide and water. However, the disadvantage is caused by a *p*H decrease in the localized region of the inflammatory response, and therefore when they do it degrades. Polycaprolactone (PCL), has a structure very similar to PLA and PGA, and it is also degraded through hydrolysis mechanisms by physiological conditions [8-10].

Chitosan, a natural polyglucosamine compound, is currently focused on more medical research. It was found that the proteins are relatively easily absorbed on the surface of biomaterials, which is a very important factor for the development of biocompatible grafts. Chitosan is used increasingly more as biomaterial because it is safe, antimicrobial, biocompatible with the human body. It has a hemostatic function, also can be used as a biocomponent and antitrombogenic in dressings [11-13].

The purpose of this work was the detailed study of morphological biodegradation and the citotoxicity effect of some experimental biomaterials for use as naturally degradable polymer products, applied in medical field, through SEM, AFM and cytotoxicity test.

Experimental part

Experimental biomaterials based on biodegradable polyacids [polylactic acid-PLA] and chitosan (CTS) with hydroxyapatite (HA) were studied. For this study were selected a series of mixtures, which include chitosan, polylactic acid (PLA) polyethylene glycol (PEG), tricalcium phosphate (TCP) and HA in varying proportions as described in table 1. There are differences between the compositions of the biomaterials with fractional PLA contents ranging from 20 to 40%.

HA was synthesized by precipitation reaction going from calcium hydroxide [$Ca(OH)_2$] and orthophosphoric acid [H_3PO_4] previously described [14]. The HA [$Ca_{10}(PO_4)_6(OH)_2$] particles obtained at low temperature (< 60°C) are monocrystalline.

The experimental biomaterials were cured at UV in air and then at 120°C, after characterized in terms of

Biomaterials	Composition [wt.%]	Ratio [%]
1	PEG +PLA+ CTS	60+40+10
2	PEG +CTS + HA	60+35+15
3	PEG +PLA +CTS + HA	60+20+5+15
4	PEG +PLA+ CTS + HA + TCP	50+20+5+15+10

Table 1
SUMMARY OF BIOCOMPOSITES CODES AND COMPOSITION

Polylactic acid (PLA)- Sigma-Aldrich, hydroxyapatite (HA) and tricalcium phosphate(TCP) - synthesized in our laboratory, Chitosan (CTS) - Sigma-Aldrich; Polyethylene glycol (PEG) 400- Sigma-Aldrich.

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topographic by atomic force microscopy (AFM- Multimode V-SPM, Veeco). The samples were biodegraded in aerobic conditions and hydro-degraded by immersion 28 h in distilled water. SEM micrographs were done for each sample, using scanning electron microscope Q130 -FEI Company. For the citotoxicity test the samples were incubated 24 and 48 hours in fibroblasts cell culture (DMEM, FCS, ATB) at 37°C, 5% CO_2. Normal human fibroblasts, which are in the third passage were grown on 24-well plates in number of 3 . 10^4/ well and incubated for 24 h in complete culture medium at 37°C, 5% CO_2. The culture medium was removed and cells were washed from two times with PBS, removed enzymatically (trypsin / EDTA) on the surface of culture and testing of viability with trypan blue. Cells were numerically investigated using Neubauer chamber. Viability is expressed as a percentage of the viable cells (not dye captures) from the total number of cells.

Viability = (normal cells/total cells) x 100

Results and discussions

Ceramic powders (hydroxyapatite and tricalcium phosphate) and biodegradable polymers [poly (lactic - glycolic acid) (PLGA) and many others] are used in repair and bone regeneration, creating a growing market for such synthetic implants [15,16]. However, materials currently used have limitations, being fragile, difficult to model or with reduced bioactivity. There are still done studies to obtain specific approval of materials and substances for using chitosan derivatives as additives or processing aids in innovative medicine [17,18].

Biomaterials based on chitosan macro porous and calcium phosphate were prepared and studied for the manufacture of a bone graft. The results revealed that the two biodegradation conditions have produced different degree of degradable surfaces after time, as can be seen in figure1.

SEM micrographs concluded that there are significant differences among the degree of biomaterials degradation. An interdependent relationship exists between the amount and chemical nature of polyacid. This result revealed in biomaterial 1 which have a high percent of PLA present a high degradation, compared with rest of the biomaterial which have HA in the composition, which decreases this process. Changes in surface characteristics of biomaterials after biodegradation are partly dependent on degradation

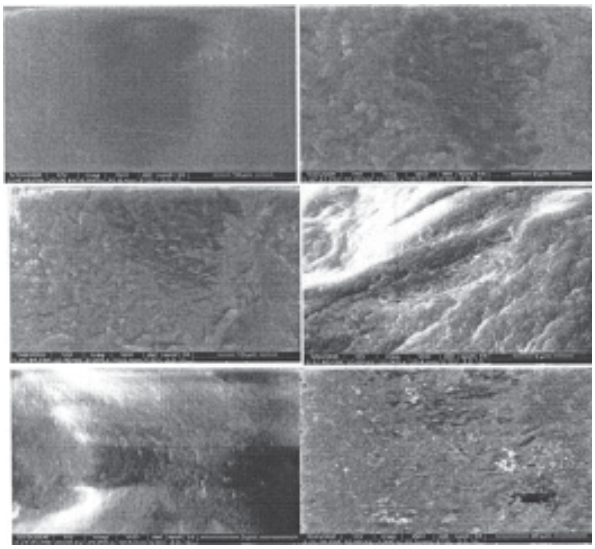

Fig. 1. SEM images of 1, 3 and 4 experimental biomaterials after 28 h of biodegradation in aerobic conditions (left) and hydro-degradation (right)

procedures. Experimental biomaterial with polylactic acid is not affected by the two different types of degradation (fig.1). Zhang and Ma achieved different porous structures based on PLA and HA for use as a material for regeneration of bone tissue [19,20]. Elastomeric copolymers, based on the D, L-lactide and ε-caprolactone (60/40) were reinforced with HA powder, aiming to influence the material properties. It was found that with increasing content of HA degradation rate of the polymer matrix decreases [21].

Biomimetic synthesis of a well organized structure as the enamel structure, has aroused particular interest because of its high potential in biomedical applications. In his work Clarkson et al [22], used sodium salt as surfactant, to modify the surface of nano rods hydroxyapatite (HA) into a hydrophobic surface. The results present that nano hydroxyapatite rods were organized into a similar structure to the enamel prisms induced by solvent evaporation.

To determine the structure of 1, 3 and 4 experimental biomaterials in which composition we have polylactic acid we investigated through Atomic Force Microscopy.

Figure 2 shows representative AFM image for 1, 3 and 4 experimental biomaterials. Obvious differences are observed on the topography of the biomaterials. In biomaterial 1 compact structure is observed, with ridges

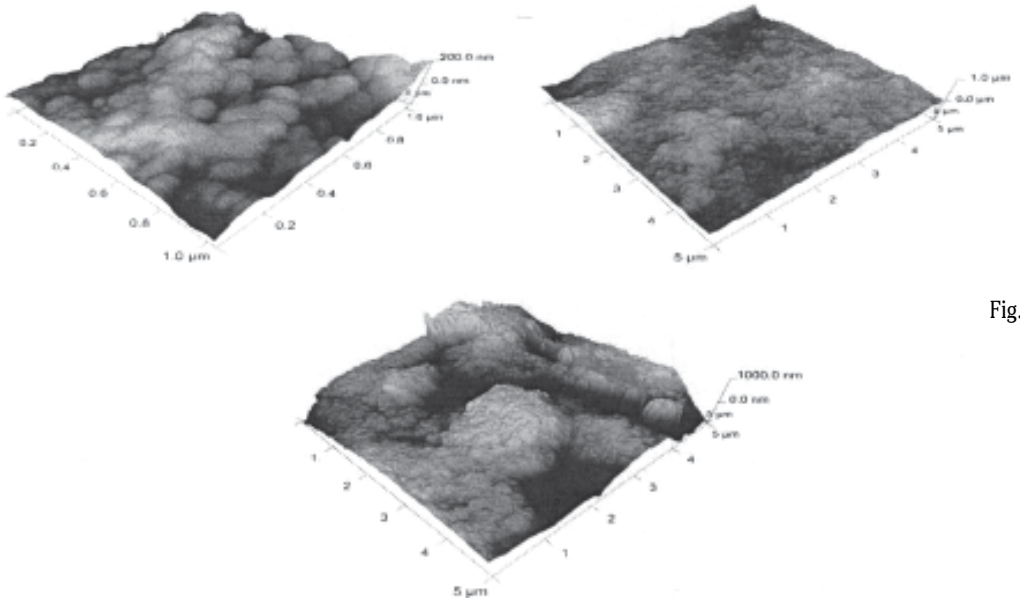

Fig. 2. AFM images for 1, 3 and 4 experimental biomaterials

Samples	Viability at 24h [%]	Viability at 48h [%]
1	83.42	89.9
2	85.05	89.2
3	84.55	88.02
4	83.89	86.9
Control sample	98.14	97.36

Table 2
VIABILITY OF FIBROBLAST CELL IN CONTACT WITH EXPERIMENTAL BIOMATERIALS

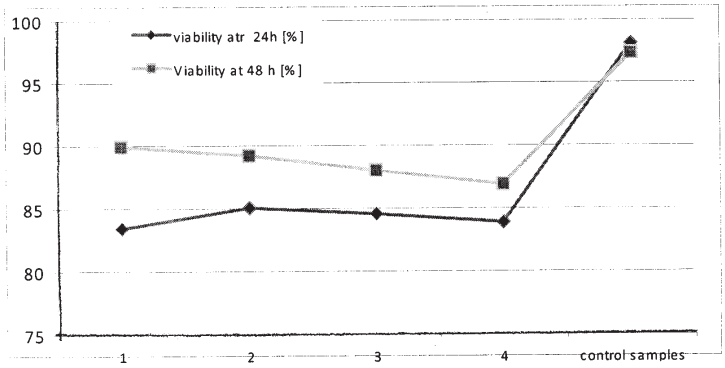

Fig. 3. Fixing and viability of fibroblast cells exposed to various samples

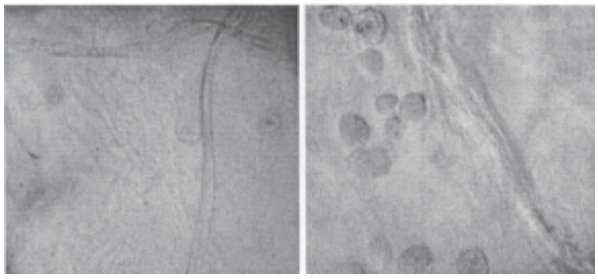

Fig. 4. Images of fibroblasts culture on samples

and grooves in the 200 nm to 1μm, and in some areas the field is even lower, ranging between 150nm-1μm. In biomaterial 4 a compact structure is observed, with ridges and grooves in 1000 nm-5μm domain, this area is found in several areas of the composite surface. Biomaterial 3 present more homogenous structures compared with 4 due to the presence of the low quantity of powder that confer a better homogeneity.

Nanostructured materials are smaller in size in the 1-100 nm, and have specific properties and functions according to the size of natural materials (eg hydroxyapatite (HA)). The development of nanofibres with nano-HA enhances (increases) the competence to manufacture scaffold that mimic natural bone tissue architecture. L.F.Charles et al [16] have developed technology for the manufacture of biomaterials based on poly (L-lactic acid) / hydroxyapatite / poly (ε-caprolactone) (PLA / HA / PCL) via a biomimetic coating process, process resulting in four stages: etching the fibber surface, depositing a covering layer of HA on PLA fibber by immersion in liquid that simulates the human body fluid (SFB), covering with PCL through a process of immersion and hot compression by casting. Incorporating strategic bioresorbable polymeric additives in hydroxyapatite cement substantively flawed in calcium can provide a low structural reinforcement and can modify the modulus of elasticity, to be closer in value to that of bone.

At the cytotoxicity test, chitosan showed low cytotoxicity according to the method used. Fibroblasts cell culture showed good adhesion to the surface of the biomaterial 4 and present close values of viability with biomaterial 1. The viability of the biomaterials increase at 48h compared with 24h, which suggest the absence of the materials cytotoxicity. All composite with chitosan retained their integrity during the experiment; their removal is possible with embedded cells, which showed good viability, so that could be a support for the fibroblasts used in therapeutic wound.

As a major inorganic component of natural bone, hydroxyapatite (HA) is a biomimetic material with good biocompatibility and bioactivity in bone tissue engineering. Hydroxyapatite has structural and compositional similarity with human bone minerals. The main applications of biomaterials chitosan/hydroxyapatite, are in the manufacture of grafts for facial trauma, as stuffing for osteolytic areas generated by tumours, or agents controlled release of drugs. Images of fibroblasts cultured films and layers on chitosan are shown in figure 4.

From examination results can be appreciated that in all cases cell viability of fibroblasts is better on tests of chitosan than the flask, and, in general, samples of chitosan with poly-ethylene glycol, respectively, hydroxyapatite present a better fixed fibroblasts cells. In conclusion, samples based on chitosan with additions can be successfully used for further studies in reparative medicine. Studies have been reported in the literature by showing preferential ability of chitosan to bind to albumin, human fibrinogen, bilirubin and immunoglobulin [6]. As control material was used polystyrene. One of the most used biocomposite membrane is chitosan-cellulose for purification of biopolymers and immunosorbtion.

Conclusions

The effectiveness on polymeric materials naturally degradable seemed to be material dependent. The term naturally degradable is intended to mean biodegradable, biocompatible, hydro-degradable, light- degradable, degradable into simple elements that do not accumulate and participate to natural cycles. The rate of biodegradability depends on the environmental conditions, also. These studies provide important information for producing a composition in the form of tablets for use in the production of naturally-degradable polymer products, a biomaterial that may find uses in the medical field as suture threads and screw for cartilage or bone fixation. Further studies will be done to determine the range of degradation time in relation with biomaterial composition, according to specific needs for clinical situations.

Acknowledgement: This paper was supported by the European Social Fund through POSDRU Program, DMI 1.5, ID 137516 PARTING.

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Manuscript received: 25.03.2015