



PREPARAREA ȘI CARACTERIZAREA GRANULELOR BIO CERAMICE POROASE DIN COMPOZITE HIBRIDE PE BAZĂ DE β -FOSFAT TRICALCIC[▲]

FABRICATION AND CHARACTERIZATION OF POROUS BIO CERAMIC BEADS BASED ON β -TRICALCIUM PHOSPHATE HYBRID COMPOSITIONS

CHRISTU ȚÂRDEI^{1*}, SORINA MITREA¹, OANA CRĂCIUNESCU², ELENA IULIA OPRÎȚA², ROXANA TRUȘCĂ³

¹ICPE-CA, Splaiul Unirii nr. 313, sector 3, 030138, București, România

²Institutul Național de Cercetare - Dezvoltare pentru Științe Biologice, Spl. Independenței nr. 296, sector 6, 060031 București, România

³SC.METAV S.A, Str. C.A.Rosetti nr. 31, sector 2, 020011, București, România

Intensive research is conducted both in understanding the phenomenon of "repair / healing of the bone" and for the development of techniques, devices and materials for effective applications involved in bone regeneration. This study is aimed at the development of a method to fabricate porous spherical tri-calcium phosphate (β -TCP) granules, which can be impregnated with a drug. Microspheres were prepared by the ionotropic gelation technique using CaCl_2 as cross-linking agent.

The microstructure and composition of ceramic microsphere were investigated by X-ray diffraction (XRD), scanning electron microscopy (SEM). In vitro cell culture tests showed that both ceramic microsphere types were highly biocompatible and favored cell growth during 72 hours of cultivation. Porous tri-calcium phosphate microspheres can be used for bone void filler, but also for drug delivery systems.

Cercetări intensive sunt realizate atât pentru înțelegerea fenomenului de "reparare/vindecare a osului" cât și pentru elaborarea de tehnici, dispozitive și materiale pentru aplicații eficiente implicate în regenerarea osoasă. Lucrarea prezintă o metodă de elaborare de granule ceramice microporoase pe bază de fosfat tricalcic (β -TCP) în scopul impregnării acestora cu un medicament. Granule ceramice pe bază de β -TCP au fost elaborate prin tehnica gelifierii ionotropice în prezența CaCl_2 ca agent de reticulare. Compoziția și microstructura microsferelor ceramice obținute au fost investigate prin măsurători de difracție de raze X (DRX) și microscopie electronică de baleiaj (MES). Testele in vitro pe culturi celulare au arătat că ambele tipuri de microsferă ceramice au fost biocompatibile și au stimulat creșterea celulară pe durata a 72 ore de cultivare. Microsferele ceramice poroase pe bază de β -TCP sunt recomandate pentru a fi utilizate ca materiale de "umplere" în chirurgia osoasă și de asemenea, ca sisteme pentru eliberare de medicamente.

Keywords: biomaterials, tri-calcium phosphate, ceramic beads, alginate, ionotropic gelation

1. Introduction

Intensive research is conducted both in understanding the phenomenon of "repair / healing of the bone" and for the development of techniques, devices and materials for effective applications involved in bone regeneration. Bone reconstruction represent a necessity in a number of clinical situations in which skeletal defects are to be restored or repaired [1, 2]. An interesting research field with medical applications is represented today by ceramics, as they can be used to obtain useful biomaterials for the production of implants [3-5]. Calcium phosphate are known able to promote new bone formation and have been already used for the repair of periodontal defects, orthopaedic and maxillofacial applications [6, 7]. Among these wide group of compounds, tricalcium phosphate (TCP) or whitlockite, $\text{Ca}_3(\text{PO}_4)_2$, is characterized by a Ca/P ratio of 1.5. Experimental and clinic investigations showed that TCP had the ability to improve new

bone formation due to its osteoconductive properties [8, 9]. Several studies have demonstrated a better conductivity, osteocompatibility and resorption rate for β -TCP than for sintered hydroxyapatite (HA) [10, 11]. In a ceramic-based bone graft material, the shape of the particles has a direct influence on bone formation. Several forms of particulate products are used as bone fillers including irregular multifaceted particles and rounded smooth granules with solid or porous structures.

Granules can be fabricating using a variety of technologies such as crushing of bulk material follow by pelletizing, spray drying, liquid quenching, or hydrothermal synthesis that yields granules of irregular or near-spherical shape [12-15]. The method to produce porous ceramic granules is based on liquids immiscibility effect, under the action of surface tension forces. Porous microspheres were prepared by extruding the slurry drop-wise into a 0.1 M CaCl_2 cross-linking solution

* Autor corespondent/Corresponding author,
Tel.: +40 21 3468 297; e-mail: christu.tardei@icpe-ca.ro

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by a needle, in combination with the porogen and ceramic powders. The ionotropic gelation technique was selected to prepare β -TCP microbeads due to its simplicity and low cost [16]. Alginate are established among the most versatile biopolymers, used in a wide range of applications, is a nontoxic, biodegradable, naturally occurring polysaccharide. Open porosity within the microsphere can be made by adding a pore former to the material. Using this method, porous granules of spherical shape are obtained in which open pores are produced by burn out of the polymeric component. Drying is an important factor in determining the degree to which water is removed from the ceramic matrix (microspheres). The rate (speed) is defined as "degree of extraction of water" and that will diminish as the process progresses. The biocompatibility of the materials was evaluated by cytotoxic effects (cell death and cell proliferation). The cytotoxicity measurements were performed on human lymphoblast cell cultures, determining the cell viability by Trypan blue method, and the proliferation rates by cell counting [17, 18]. Many factors affect the solubility of calcium phosphate based materials including the method of preparation, phase content, density, the extent of ionic substitutions into the apatite lattice and microstructure. Their dissolution behaviour varies widely [19]. The dissolution rates of β -TCP microspheres are different due to the different surface density and crystallinity of them. Dissolution of microspheres was analyzed by measuring the pH variation in physiological conditions, in distilled water, at different times between 0-4 hrs, and respectively at 1-4 days.

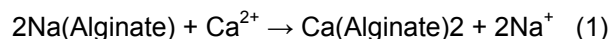
This work describes the preparation and characterization of microspheres intended to be used as a biomaterial for bone regeneration or as drug-delivery matrices. The method to produce porous granules is based on liquid immiscibility effect, by the ionotropic gelation technique, using CaCl_2 as cross-linking agent. The main objective of this paper was the evaluation of the physico-chemical, morphological and biological properties of two variants of β -TCP microbeads. Granules from β -TCP powders of 500 – 3,000 μm diameter were produced.

2. Materials and experimental procedures

2.1. Materials

Ceramic powders, produced in our laboratory, were prepared via solid-state reaction using a methodology previously described [20]. Briefly, β -TCP powders were prepared by sintering the stoichiometric mixture of $\text{NH}_4\text{H}_2\text{PO}_4$ and CaCO_3 (2:3 molar ratio) at 1100°C. A heating rate of 5°C·min⁻¹ and a cooling rate of 10°C·min⁻¹ were used. Finally, the powder was wet-milled for 4h to gain suitable particle size distribution and specific surface area. The as-obtained powder ($d_{50} \approx$

1.86 μm) was used as starting powder for this work. Alginic acid sodium salt from brown algae and calcium chloride were purchased from Fluka. All reagents were of analytical grade and used as received. One of the most important and useful properties of alginates is the ability to form gels in the presence of divalent cations, especially calcium, resulting in formation of three-dimensional network; the reticulation process consists of the simple substitution of sodium ions with calcium ions, as in the following reaction:



Monovalent cations and Mg^{2+} ions do not induce gelation while Ba^{2+} and Sr^{2+} ions produce stronger alginate gels than Ca^{2+} [21]. Furthermore, by selecting of the type of alginate and coating agent, the pore size, the degradation rate, and ultimately the release kinetics can be controlled. The sodium alginate water solution (1 - 3%w/v) was prepared by dispersing the sodium alginate in de-ionised water under continuous stirring for 1 hour. The dispersion was sonicated for 30 min to remove any air bubbles that may have been formed during stirring process.

2.2. Preparation of β -TCP porous microspheres

The starting material was a finely dispersed β -TCP powders with a Ca/P ratio of 1.5, and a specific surface area of 5.85 m²/g (BET method). Suitable amount of ceramic powder was dispersed in deionised water by 15 min. ultrasonication. Ultrasonication was necessary to avoid agglomeration of ceramic powder and to achieve proper dispersion. The powder was mixed with a biopolymer solution and the suspension was added, using a magnetic agitator, to a dispersion fluid at room temperature. The best *dispersant* was selected on the basis of electrophoretic, sedimentation, and viscosity measurements. β -TCP in water was mixed with polymer solution under agitation. The agitator speed varied from 200 to 600 rpm. The resulted homogeneous dispersion was extruded in to 500ml of calcium chloride (1.5% w/v) solution through hypodermic syringe with needle tip (20G) and stirred at 200rpm using magnetic stirrer. Microspheres were prepared by the ionotropic gelation technique using CaCl_2 as cross-linking agent, according to the procedure depicted in Figure 1. A Gelation time of 30 min. was allowed to complete the curing reaction and produce spherical and rigid microbeads. The beads so prepared were collected by decantation, washed with distilled water and dried.

For experiments were made different compositions with ceramic powder /polymer solution ratio ranging in the 0.1 ...0.5 domain. Experimental parameters include concentration of alginate and of cross-linking solutions, temperature

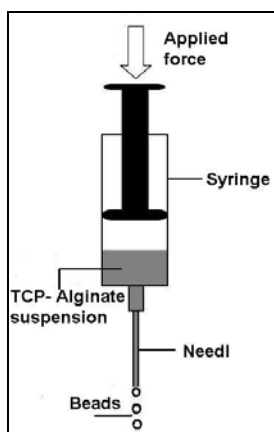


Fig.1 - Schematic representation of the alginate- β -TCP composite microsphere extruding using syringe technique / Sistem de picurare ("extrudere") a amestecului de soluție de alginat și pulberea ceramică cu ajutorul unei seringi cu ac.

of β -TCP calcination, solid to polymer ratio, stirring speed, surfactant types, etc. Were developed suspensions with different rheology with an increasing ratio between the amount of ceramic powder and aqueous solution of sodium alginate. Were developed two types of microspheres, further noted AD and S. So, type AD microspheres were prepared by direct mixing of the components while, S-type microspheres were prepared by making initially a stable β -TCP suspension, after which they were introduced the remaining components. *Open porosity* within the microsphere are made by adding a pore former (cellulose microcrystalline as powder, from Merck) to the material. The specimens were dehydrated through a series of aqueous solutions containing ethanol with increasing concentration up to 100% (v=v). The microspheres produced were measured in terms of average diameter at different times after drying at 24h and 72h, and finally after sintering at 1000 to 1150°C.

2.3. Characterization

2.3.1. Phase composition of the β -TCP powders and microspheres

The phase composition of the calcined β -TCP powders and microsphere samples were characterised by X-ray powder diffraction (XRD) method, using $\text{CuK}\alpha$ radiation, type Bruker-AXS, D8 ADVANCE high-resolution diffractometer, over the 2θ range of 20 to 60°. The identification was carried out by comparing the peak positions and intensities with those in the joint Committee on powder diffraction Standards (JCPDS, 09-0169) data files.

2.3.2. Density and porosity measurements

The Archimedes water immersion technique was found to be the best in measuring density and open porosity. Specimen density was measured on each sintered specimen using the Archimedes principle using SORTORIUS density

measurement kit. The measurements were carried out in distilled water. These were then compared with the theoretical densities for β -TCP (3.07 g/cm³)

2.3.3. Morpho-structural analysis

Morpho-structural analysis by optical microscopy was performed by visualization with Carl Zeiss Jena microscope NU2 type, with DinoLite digital attached camera and image acquisition software. Photographs of samples were made in direct reflected light to increase the magnification to 300X. The morphological properties of beads surface and the internal structure characteristics of beads (e.g. porosity, pore size, surface area) were evaluated using scanning electron microscope (SEM), using scanning electron microscope type FESEM-FIB Auriga model produced by Carl Zeiss Germany.

2.3.4. Biocompatibility and bioresorption assessments, in vitro tests

2.3.4.1. Cytotoxicity experiment

In order to evaluate possible release of toxic compounds from ceramic microspheres, the extract method was used, according to SR EN ISO 10993-5 for testing medical device cytotoxicity. Cell suspensions were seeded at a density of 5×10^4 cells/well in 24-well culture plates, allowed to adhere by culturing in MEM containing 10% FBS and incubation in a humidified 5% CO₂ atmosphere, at 37°C, for 24h. Then, the culture medium was replaced with microsphere extract in the same medium, in three different concentrations (1, 5, 10 mg/ml). Culture plates were incubated in 5% CO₂ atmosphere, at 37°C, for 24h, 48h and 72h, respectively. Untreated cells served as control. The influence of microsphere extracts on the activity of dehydrogenases from cell mitochondria was evaluated by MTT assay [17]. Briefly, after each period of cultivation, the culture media was removed and a solution of 0.25 mg/ml methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added in each well. After incubation at 37°C for 3 h, the formazan crystals formed in viable cells were dissolved in 1 ml isopropanol. Absorbance was measured at 570 nm using a Jasco V-650 spectrophotometer and was proportional to cell viability. Results were reported as percentage from the control value, considered as 100% viable cells.

2.3.4.2. Bioresorption test

Hydrolytic stability test (bioresorption) was achieved by pH measurements. Dissolution of microspheres of β -TCP was analyzed by measuring the pH variation in physiological conditions, in distilled water. Samples of β -TCP microspheres were dissolved in distilled water, pH 5.85, in a concentration of 1 mg/ml, with mild shaking at room temperature (25°C). Measurement was performed using a pH meter

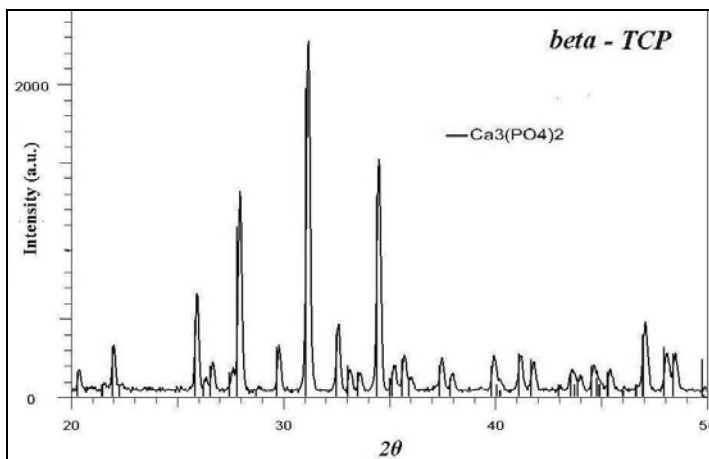


Fig.2 - XRD spectrum compared with the JCPDS card 09-0169 of the synthesized β -TCP powder / Difractograma compusului β -TCP raportată la fișele JCPDS, card 09-0169.

CONSORT C - 861 (Belgium) at different times between 0 and 4h, and 1-4 days, respectively. The experiment was performed in triplicate. The data obtained are expressed as percentage of control and mean of 3 determinations \pm standard deviation (SD).

2.3.4.3. Statistics

The results were reported as mean \pm standard deviation (SD). Statistical analysis was performed using Student t-test and statistical significant differences were considered at $p < 0.05$.

3. Results and discussions

3.1. X-ray diffraction and specific surface area for β -TCP powders

Powders sintered at different temperatures present in terms of composition, β -TCP as a single phase. The XRD pattern are shown in Figure 2 and no extra peaks were found.

3.2. Preparation of β -TCP-alginate microspheres

The spherical geometry of granules is reflected in Figure 3. Diameter of the bead was about 2500 μm , and micropores were observed on the surface. The analysis revealed that microspheres were homogeneous, in terms of size and shape, and no evidence of cracks was found. Its shape is spherical but sometimes skewed at the low concentration of sodium alginate, less than 1.75% (w/v). Increasing the concentration of CaCl_2 to 3% (w/v) should increase the porosity of beads, while using 0.5% (w/v) of CaCl_2 gives weak gel due to insufficient cross-linking of alginate. Deformed beads were also produced if the ratio of ceramic powder to polymeric solution was increased above certain limits.

The specimens were dehydrated through a series of aqueous solutions containing ethanol with increasing concentration. For experiments using a water extraction algorithm from 50% alcohol solution or pure alcohol, the weight difference measurements at predetermined intervals (4h, 16h, 48h and 72h). Main results are presented in Figure 4.

Microspheres type S in which the amount of water used for preparation is greater, by extraction with alcohol showed the greatest water extraction.

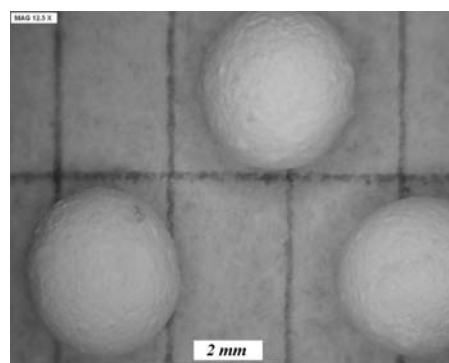


Fig.3 - Microscopy for ceramic microspheres based on β -TCP, obtained by ionotropic gelification technique / Microscopie optică pentru microsferile ceramice pe bază de β -TCP obținute prin gelifiere ionotroptică.

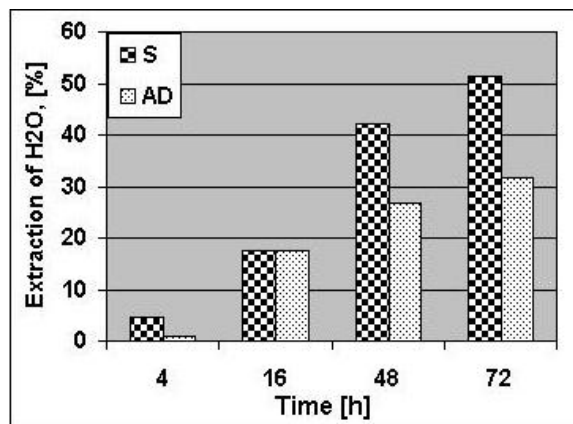


Fig. 4 - Dynamic drying process by means of water extraction in the time interval 4-72 hours, for microspheres type AD & S / Dinamica procesului de uscare cu extracție de apă în intervalul de timp 4-72 ore, pentru microsferile tip AD și S.

3.3. Preparation of β -TCP, porous beads

Open porosity within the microsphere are made by sintering at a temperature less than that required to fully densify the material, by changing the ceramic-to-polymer solution ratio, or by adding

a pore former to the material. Figure 5 shows the influence of the factors mentioned above on the level of porosity for the obtained microspheres. An obvious influence is the growing of polymer component; moreover, further addition of ~ 10% wt. porogen material cause an increase in the level of porosity to more than 50%.

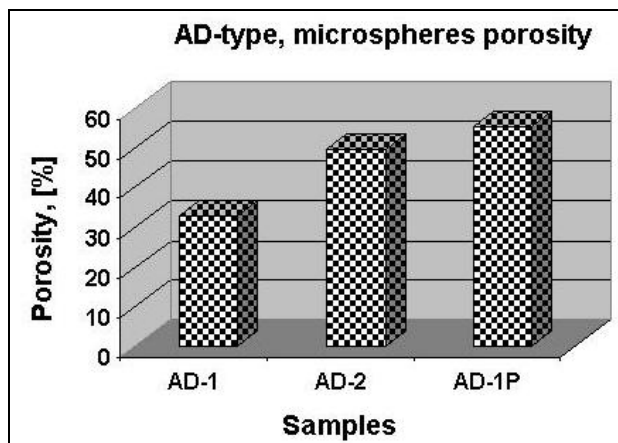


Fig.5 - Influence of the porogen addition and the polymer/ceramic powder ratio on the total porosity of the AD-type microspheres / *Influența adaosului de porogen și a raportului polimer/pulbere ceramică, asupra porozității totale pentru microsferile elaborate, tip AD.*

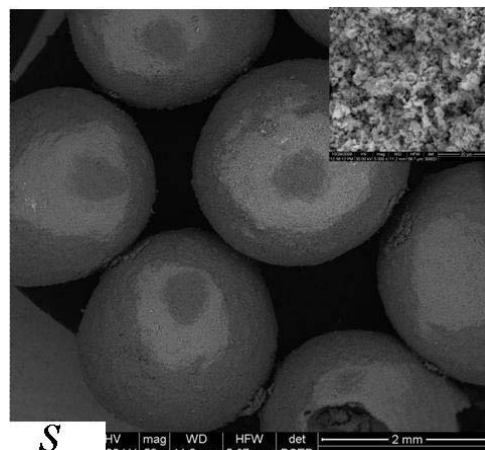
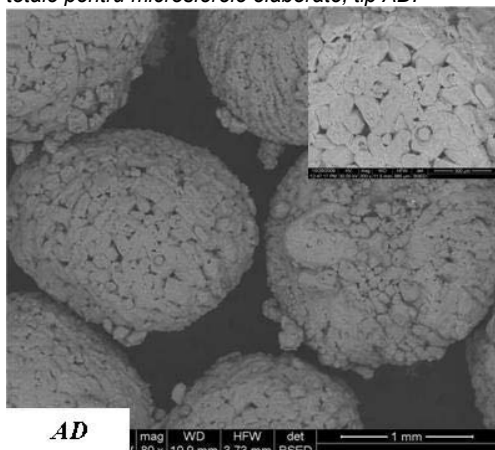


Fig. 6 - SEM photographs of the two types of β -TCP beads, with intra- and intergrain porosity / *Microscopie electronica (SEM) pentru cele doua tipuri de microsferice ceramice pe baza de β -TCP, prezentand porozitate inter și intragranulară*

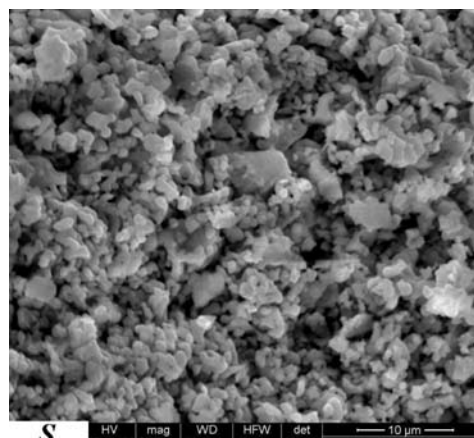
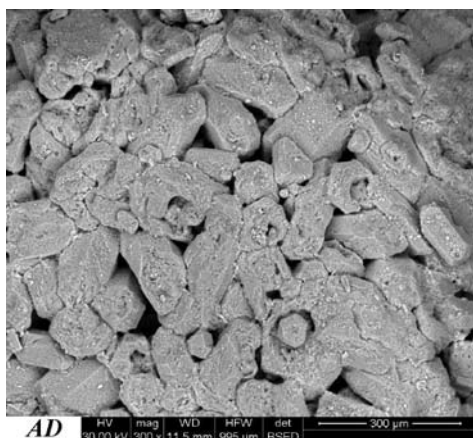


Fig. 7- SEM images shows that powders AD are constituted of agglomerated elementary particles due to diffusion bonding particles; powders exhibit grains with a significant size which are strongly bonded ones with the others / *Imaginile de microscopie electronica SEM arată că pulberile AD sunt constituite din particule elementare aglomerate unite prin difuzie; pulberile prezintă granule de mărime semnificativă unite puternic între ele.*

Figure 6 and 7 shows micrographs (SEM) for the two types of microspheres, and outlines the morphology and microstructure of microspheres. The surfaces of the AD-microspheres were coarser than the surface of the S-microspheres.

The microstructural analysis of the β -TCP microspheres shows pores which are preferentially located on the grain boundaries. Pores with sizes of about 45 μ m dominated, their relative content in the total number of open pores reached ~ 56%. The burning out of biopolymers is accompanied by the formation of fine pores (intra-pores) in granules, whereas the formation of inter-granular interconnected pores (inter-pores) is determined by the packing of granules. The proportion and particle size of the pore-forming agent influences the porosity and the pores structure.

3.4. Biocompatibility and bioresorption of β -TCP microspheres, in vitro tests

Figures 8, 9, the values of cell viability were higher or at least similar to that of control cells (100%), for all tested concentrations. Both types of microspheres (AD and S) exhibited a significant effect on cell growth. The highest values of cell

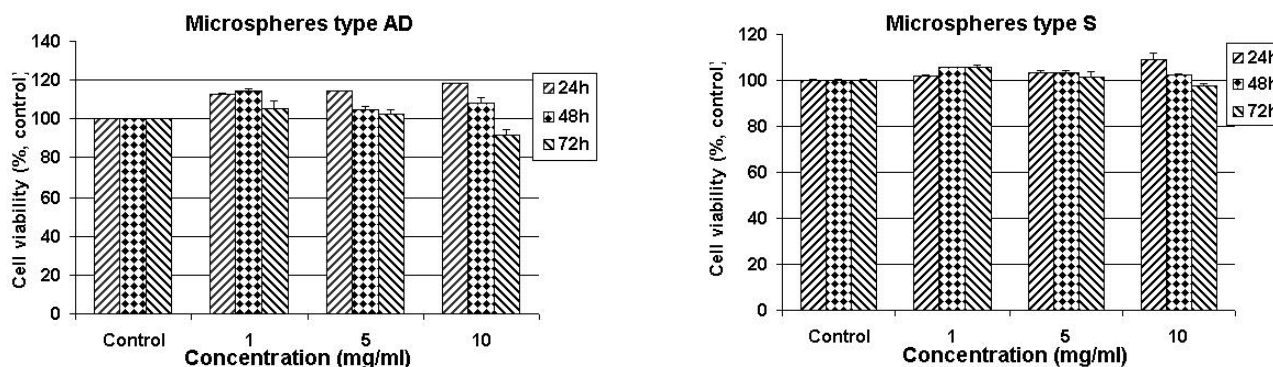


Fig. 8 și 9 - Variation of fibroblasts viability cultured in the presence of different concentrations of β -TCP microspheres (AD & S), at different times of cultivation. Values are mean of 6 determinations \pm SD. The cell viability of control culture was considered 100% / Variația viabilității fibroblastelor cultivate în prezența diferitelor concentrații de microsferă ceramică pe bază de β -TCP (AD și S), la timpuri diferite de cultivare. Valorile reprezintă media a 6 determinări \pm SD. Valoarea viabilității celulare a culturii martor a fost considerată 100 %.

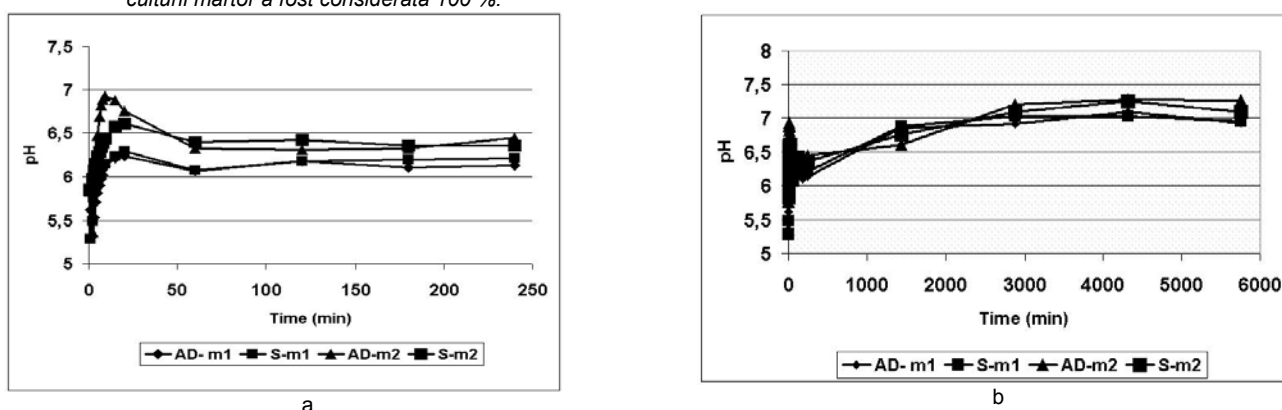


Fig. 10 - The pH variation for two samples (ceramic microspheres AD & S), between 0-4 hours (10a), and that for the period 0-4 days (10b) / Variația pH-ului pentru 2 probe (microsferă ceramică), în intervalul 0-4 ore (10a), și respectiv pentru intervalul 0-4 zile (10b).

viability (115-120%) were recorded for microspheres type AD (1-10 mg/ml) (Fig. 8) and microspheres type S (10 mg/ml) (Fig. 9), at 24h of cultivation in the fibroblast culture. These values were significantly higher ($p < 0.05$) than those of the control culture (100%). These results indicated that ceramic microspheres are bioactive materials, able to control and stimulate the cellular activity of fibroblast-type cells.

The dissolution behavior of the synthesized powders in the Ringer's solution was shown in Figure 10 a/b. The pH values differ due to preparation conditions of the materials and experimental conditions of the soaking tests. The pH of the solution increased rapidly up to ~ 7.5 and remained constant thereafter. For both tested samples of ceramic microspheres has been a rapid degradation in the first 20 minutes when we observed a large variation in pH, followed by a minimum variation of pH in the next 4 hours. Further, during 4 days was a slow increase of the pH value for both samples, explained by controlled release of positive ions of calcium and negative ions of phosphate from ceramic microspheres that in time stabilizes the neutral pH value ($\text{pH} \sim 7.0$).

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4. Conclusions

Powders and porous microspheres based on β -TCP were obtained and tested. The microspheres were prepared by the ionotropic gelation technique using CaCl_2 as cross-linking agent. The size of spherical particles of β -TCP with alginate was depended on the ceramic-to-polymer ratios, stirring rate, and the concentration of cross-linking solution. Cross-linking of β -TCP/alginate microspheres for at least 15 minutes was necessary for the formation of sufficiently strong microspheres. It was observed that reducing the needle diameter could lead to smaller beads. The shape of wet beads was influenced by the distance between the top of the needle through which the dispersion was dropped and hardening solution, as well as the stirring speed of the hardening solution. Drying technique could influence several bead characteristics such as size, shape, mechanical properties and morphology of beads including surface as well as internal structure. We can conclude that calcium phosphate porous microspheres prepared by ionotropic gelation technique are effective for development of drug delivery matrices or as fillers in bone surgery. Additional research is still required to determine the optimal type of carrier material, its loading and release capacity for particular clinical indications.

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