LAYERED SHAPED ALGINATE HYDROGELS FOR SOFT TISSUE ENGINEERING BASED ON CHEMICAL CONTROL OF THE CROSSLINKING RATE

D. DIMONIE[a*], R. GABOR[a], V. MITRAN[b], E. VASILE[c], R.TRUSCA[c], M. PETRACHE[a], S. DONCEA[a], R. C. FIERASCU[a], I. FIERASCU[a], C. NICOLAE[a]
[a]National Research and Development Institute for Chemistry and Petrochemistry, Bucharest, Romania
[b]University of Bucharest, Faculty of Biology, Department of Biochemistry and Molecular Biology Bucharest, Romania
[c]SC Metav-CD SA, Bucharest, Romania

The reactants concentrations and reactions conditions that ensure the obtaining of hydrogels with homogeneous morphology and physical and dynamo-mechanical properties optimal as 3-D scaffolds for soft tissue engineering were found. The obtained hydrogels are transparent and homogeneous, have 98 ± 2 % hydration degree, $96 - 98$ % porosity, good transport properties, uniform microstructure with comparable pores in sizes and shapes and handling consistency. The new hydrogels have elastic properties in the range of $200 - 3400$ Pa meaning similar with those of soft tissue like mammary gland, lymph node, brain, liver, etc. However the fibroblasts survival rate in the new acquired hydrogels was not the highest. The improvement of the hydrogels biological properties can be achieved based on a method to slow down the gelling rate and to better remove the air bubbles residual content, as well as possible unreacted $CaCO_3$ and /or GDL traces. If the crosslinking rate is smaller, the hydrogels can result with enlarged pores.

1. Introduction

The primary function of the scaffolds in tissue engineering is to provide a 3D template to introduce the progenitor MSCs to the specific site of interest and to provide interim mechanical stability for tissue growth and integration [1, 2]. This support must be biodegradable at a comparable rate to extracellular matrix production, biocompatible, non-toxic to the cells, easy to manufacture, able to transfer nutrients and metabolites to the cells, to remove the wastes without adverse effects on the cells and to have sufficient mechanical integrity and strength to withstand manipulations associated with testing in vitro and in vivo implantation and existence [1, 3]. Further, the material should promote endogenous cell infiltration, promote angiogenesis. Soft tissue engineering studies are conducted on organs such as liver, lung, muscles, skin, nerves, blood vessels, cornea, vagina, heart valves, trachea etc.

The alginate is a polysaccharide composed from blocks of (1-4) β-D-mannuronic acid (M) and α-L-glucuronic acid and can be found in brown algae and bacteria [4, 5]. In contrast to most other polysaccharides, alginates do not contain repeating monomer sequence units along the polymer chains. Hence, there is a vast compositional heterogeneity among alginates isolated from different organisms and even among specimens obtained from different parts of the same organism [6]. Sodium alginate can be used to obtain hydrogels for tissue engineering as it shows previously described properties needed for the use in this area.

Alginate gelation can be achieved by covalent or ionic crosslinking. Ionically crosslink takes place in the presence of divalent cations that interact with carboxylic groups of the

---

*Corresponding author: ddimonie@yahoo.com

glucuronic acid units from the alginic acid, forming ionic bonds and "egg - box" structures [7]. The properties of the resulted hydrogels depend by the alginate type, calcium ions concentration, temperature etc. [5, 7, and 8]. The most used divalent cations are: Ca^{2+}, Ba^{2+} and the most studied crosslinker ions suppliers are: $CaCl_2$, $CaCO_3$, $CaSO_4$, $Ca_3(PO_4)_2$, CaEGTA, $CaCO_3$-GDL, GDL-$CaCO_3$-$CaSO_4$ [9-11]. In order to ionically crosslink the alginate with divalent cations, the most used methods are dropping an alginate solution [12] into a $CaCl_2$ one or a $CaCl_2$ solution in another alginate one [13]. Due to the high reaction rate of the calcium ions with guluronic blocks of alginic acid, which practically cannot be controlled, beads irregular in shape, rigid, difficult to be modelled with small pore sizes are formed [14, 15]. Hydrogels thus obtained are usually used for bone [16-19], or cartilage [7, 18, 20] tissue regeneration. That is why the known literature data regarding the alginate ionically crosslink generally refer at those reaction conditions and reactants concentration that lead to hydrogels with properties adequate to bone and cartilaginous tissues engineering. High polymer and calcium ions concentrations (1.5 – 3 %) were generally investigated, so that a gelation rate by 3 h was achieved only at 5 °C. At physiological temperature (37 °C) the best gelation time was by 50 minutes [5].

Material properties appear to be relevant to the normal development of tissue during embryogenesis and growth. The mechanical properties of the substrate on which or within which cells are placed can have as large an impact as chemical stimuli on cell morphology, differentiation, motility and commitment to live or die. The hydrogels mechanical properties must be similar with those of the living tissue and their level depends on the tissue type. The stiffness of native cartilage tissue is ranged between 089 MPa-2.22 MPa [21, 22] and those for bone regeneration have higher values, for ex. by 12 GPa in case of trabercular bone [23].

The scaffold microstructure guide cellular organization, cells proliferation and matrix production. Since the porous channels within the hydrogels are not straight and the sizes of the pores are not uniform these issues are generally included in a factor generally known as tortuous path [24]. The microstructure and the average pore size of the hydrogels greatly affect the growth and penetration of cells in the 3-D structure of hydrogels [25].

The aim of the study was to achieve, at physiological temperature (37 °C), in a gelation time by approx. 3 h, layered shaped interconnected 3-D porous structures by hydrogel type, with elastic properties and pores size designed for adipose tissue engineering based on a method to chemical control the migration of divalent cationits which ionically crosslink the sodium alginate. The study has focused also on the correlation of the obtaining conditions with the hydrogels properties, considering inclusively the growth and proliferation capacity of fibroblasts embedded in the polymer solution, before crosslinking.

2. Experiments

The hydrogels obtaining were carried out with 0.5 – 1.5 % (w/v) sodium alginate (Fluka – 7120) solutions in distilled water, 0.1 % - 0.2% $CaCO_3$(Scharlau) and 0.37 % -0.74 % GDL (Merk). The hydrogels were made also in culture medium (Eagle's minimum Essential Medium – MEM, Sigma Aldrich Co., M 3024), in two alternatives, with and without serum. For each studied reactants concentrations, all reagents were mixed together. The reaction product, with homogeneous appearance, was pipetted into wells of a Multiwell culture plate in a volume that assured the obtaining of 4-5 mm height hydrogels. The plates were kept for 3 hours at 37 °C, in an oven. After crosslinking the hydrogels were two times washed, for 30 min., with distilled water.

CCD 1070SK fibroblast cell line (from ATTC collection, CLR-2091) was maintained and propagated in MEM (Eagle's Minimum Essential Medium) (Sigma- Aldrich. Co) culture medium supplemented with 10% fetal bovine serum (FBS, Gibco) and incubated at 37°C in a humidified atmosphere of 5% CO_2. The growth media were changed every third days, until cells reached the confluence stage. The alginate solution that was used for fibroblast embedding was prepared under sterile conditions in a laminar flow hood from 1.5% (w/v) sodium alginate in MEM culture medium containing 10% fetal bovine serum (FBS - Gibco, 10091 – 148) sequentially mixed with sterile $CaCO_3$ and GDL powders at concentrations of 0.14% and 0.48%, respectively. At 80% confluence, the fibroblasts were detached from the cultured dishes using 0.25% Trypsin - 0.03%

Ethylenediaminetetraacetic acid (EDTA) (Sigma – Aldrich Co., E-6511), counted and mixed with the alginate solution at a final concentration of 4 x 10^5 cells/ml. The cell-alginate mixture (1 ml) was distributed into Petri culture plates (8 cm^2, Nunc) and allowed to crosslink for 75 minutes at the room temperature. After crosslinking, the alginate matrix was washed two times and covered with 2 ml MEM complete medium. The fibroblast morphology post-alginate embedding was observed by phase contrast microscopy.

Hydrogels chemical structure was analyzed with Fourier Transform Infrared Spectroscopy (FTIR), Energy Dispersive X Ray Fluorescence (EDXRF) and Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). For FTIR analysis was used an Perkin Elmer Spectrum GX device, working in ATR mode, in the 4000-600 cm^{-1} wave number range. The EDXRF analysis were performed using a PW 4025 MiniPal 2 spectrometer (PANalytical) equipped with a Si(PIN) detector with resolution of 150 eV at 5.89 keV, MnK_α line. The technique is non destructive and does not require sample preparation procedures. Working parameters are usual for qualitative assay [26]: time 300 sec, helium atmosphere and no filter. The analysis was performed at 20 kV and automatic amperage. For ICP-AES analysis samples were mineralized with nitric acid (80ml) and hydrogen peroxide (10ml) by heating on sand bath. ICP-AES on a spectrometer Liberty 110, Varian with following characteristics: monoelement measurement, detection limits higher than 0.4 mg/kg, V-Grove nebulizer, Fassel Torch and Czerny Turner monochromator [27].

Hydrogels gelation rate was estimated based on consistency. Consistency was considered as the crosslinking density at which the hydrogels keep their shape, volume, storage modulus and stiffness for 20 min., at 1 Hz. If the condition is fulfilling the reactants mixture does not flow when the vial was tilted at an angle and the hydrogels can be manipulated. The storage modulus and the stiffness were recorded on a A DMA Q800 device, working in air at a frequency of 1.00 Hz and using cylindrical hydrogels by 2.3 – 4.3 mm thickness and : 23.55 mm diameter. Method: Frequency sweep; Procedure: Equilibrate at 37 °C, Isothermal for 300 min. Repeat segment 4 for 99 times. The averages and the standard deviations of triplets were reported (mean ± standard deviation).

Hydrogel stability in physiological conditions was estimated by hydrogels keeping, in oven, at 37 ^{0}C, for 76 hours, in the wells of the culture plaque, and periodically the value of the storage modulus and stiffness were registered on the DMA Q 800 device.

Hydrogel porosity [28]. Both the mass (M) and the volume (V) of the prepared scaffolds were measured. Vp represents the polymer volume. The porosity was calculated using the equation (1). At least three measurements were performed for each sample and the mean values and standard deviation are reported (mean ± standard deviation).

$$\text{Porosity, } [\%] = \frac{V - V_P}{V \times 100} \qquad (1)$$

*Homogeneity [*29] represents the differences in dry to wet weight ration of subsequent slices of the sample.. At least three measurements were performed for each sample and the mean values and standard deviation are reported (mean ± standard deviation).

Swelling behaviour[30]. *Degree of swelling* (SD), defined as the differences between the weight of the swollen hydrogel sample at time t, m_t, and the weight of the hydrogel sample, m_0, divided by the hydrogel weight, was calculated according to Eq. (2) and determined as a function of time, to constant variation. The SD was determined in deionized water. The equilibrium degree of swelling, SD_{Eq}, is the degree of swelling of the swollen hydrogel at equilibrium, i.e., the hydrogel sample which had reached constant mass, m_{eq}. At least three swelling measurements were performed for each sample and the mean values are reported (mean ± standard deviation)..

$$SD[\%] = \frac{(m_t - m_0)}{m_0} \times 100 \qquad (2)$$

Hydrogels microstructure was analyzed using a scanning electron microscope Quanta INSPECT F equipped with electron field emission gun - EFG with a resolution of 1.2 nm was used. Lyophilisation was performed in a CHRIST ALPHA 1-2 LD plus freezer in the following

conditions: main drying at -42.9 ° C and 0.091 mbar and finally drying at -43.2°C and 0.012 mbar. It was considered the hydrogels diameter (D) and the aspect ratio (A). The pore diameter was calculated by using the formula $D = \sqrt{l \times w}$, where l represents the longest distance across the pore in question (length) and w is the longest length across the pore perpendicular to l (width)[3] [31]. The aspect ratio of pore was calculated by means of the relationship $A = l/w^3$ [31]. Obviously, a value which equals to 1 or very close to 1 would equate to a circular or square pore. Values lower or higher than 1 represent the pores of increasingly elliptical (oval) nature (rectangular pores) [31]. The averages and standard deviations for 50 pore measurement in each case are reported. The statistical analysis was done with Origin Pro 8 software.

The survival fibroblasts embedded into alginate hydrogels was evaluated by measuring lactate dehydrogenase (LDH) activity released into the culture medium. Samples of 100 μL culture media were taken at 3 and 6 days of culture and a LDH test was performed by using Cytotoxicity Detection Kit (TOX-7, Sigma Aldrich Co.) according to the manufacturer's protocol. Data are presented as the average of three replicates (mean ± standard deviation).

Cell viability of the fibroblasts embedded into alginate hydrogels was quantitatively determined by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma Aldrich Co, M 2185)) test [32]. After 3 and 6 days, the culture medium was replaced with freshly prepared MTT solution (1mg/mL MTT in serum free MEM) and incubated at 37°C for 20 h. Water-insoluble purple formazan crystals formed in the viable metabolically active fibroblasts embedded in alginate hydrogels were solubilised with isopropanol (Fluka, 59300) for 2.5 h under continuous shaking and the absorbance was measured at 550 nm using a microplate reader (Thermo Scientific Appliskan).

Cell viability and number were determined by hemocytometer using Trypan blue (Sigma Aldrich Co., T 8154) exclusion test. The method is based on the principle that live cells possess intact membranes that exclude trypan blue, whereas dead cells do not, and therefore viable cells will have a clear cytoplasm whereas non-viable cells will have a blue cytoplasm when are observed under a phase contrast microscope (Olympus IX 71) [33]. To recover the embedded cells, alginate hydrogel was solubilized into a solution containing 55 mM sodium citrate, 5mM EDTA (Sigma Aldrich Co., E 6511) and 0.15mM NaCl. Then aliquots were taken from each plate and mixed 1:1 with a 0.2% w/v trypan blue solution (Sigma-Aldrich Co.) for cell counting under phase contrast microscope. MTT and LDH assays were performed in triplicates and statistically analyzed using Graph Prism software. All values are expressed as mean value ± standard deviation and differences at $p \leq 0.05$ were considered statistically significant.

3. Results

3.1 Hydrogels formation After crosslinking for 3 hours, in oven, at 37°C, layered shaped calcium alginate hydrogels with different level of handling properties were acquired (Fig.1). The infrared spectroscopy indicated that the organic part from the hydrogel FTIR spectrum is alginate (fig 1a). Because the spectral differences between sodium alginate and calcium alginate there are placed in unspecific spectral areas (600 – 400 cm^{-1}), the FTIR is not a good method to emphasize the conversion of sodium alginate into calcium alginate. To confirm the chemical structure, the hydrogels and sodium alginate were comparatively analyzed through EDXRF and ICP-AES. These two methods denoted that the inorganic part from hydrogels is mainly represented by calcium cationites. The qualitative presence of calcium as major element was detected through EDXRF (fig 1b). An increase of calcium content from 1.05% in sodium alginate to 15.33% in hydrogel and also a decrease in sodium content from 30% in sodium alginate to approximately 12% in hydrogels, by ICP-AES were measured.

The consistency of the obtained hydrogels depends by the ratio between the reactants and the nature of the gelling environment. At the same reaction conditions, an important difference, in terms of handling consistency, depending on the nature of the reaction medium was observed. At the same reactants concentration, the handling consistency of the hydrogels made in MEM (with and without serum) was much smaller. At small reactants concentrations it is possible to results gelatinous hydrogels that cannot be handled. In culture medium, hydrogels with handling

properties comparable with those of the hydrogels crosslinked in distilled water, only at high reactants concentrations were obtained. If the hydrogels are not washed to stop the reaction, after crosslinking, the storage modulus continues to increase even for 120 h (Fig.2) or more. Storage modulus (fig 3a) has low and comparable values at 0.5 - 1% sodium alginate, 0.49% DGL and 0.1 - 0.14% $CaCO_3$. This property exceeds 2 kPa at 0.49 % DGL and 0.2 % $CaCO_3$. Storage module remains below 2 kPa even if the concentration of sodium alginate increases at 1.5% and GDS is ranged between 0.37 and 0.49%, and the $CaCO_3$ is ranged into 0.1 - 0.14 % interval. This property exceeds even 3 kPa at 1 - 1.5% sodium alginate, the 0.74% GDS and 0.2% $CaCO_3$. Stiffness (fig 3b) is low and have almost same values of about 50 N/m if the concentration of sodium alginate is 0.5 - 1%, those of GDL 0.49 - 0.74 % and $CaCO_3$ 0.14 - 0.2%. This property doesn't have higher values even if sodium alginate concentration is 1.5%, GDL 0.49% and $CaCO_3$ 0.1%. However, it exceeds 100 N / m when the concentration of sodium alginate is 1.5% but GDL is 0.49-0.74% and $CaCO_3$ 0.2%. The influence of calcium ions on the hydrogels elastic properties becomes relevant at high sodium alginate concentrations (1.5 %). Fig.3c,d show that, at this polymer concentration and 0.4 g/l Ca^{2+} , the storage modulus is 1000 Pa and stiffness is by 40 N/m. If the Ca^{2+} increases at 0.8 g/l the storage modulus becomes 3250 Pa and stiffness reaches 160 N/m.
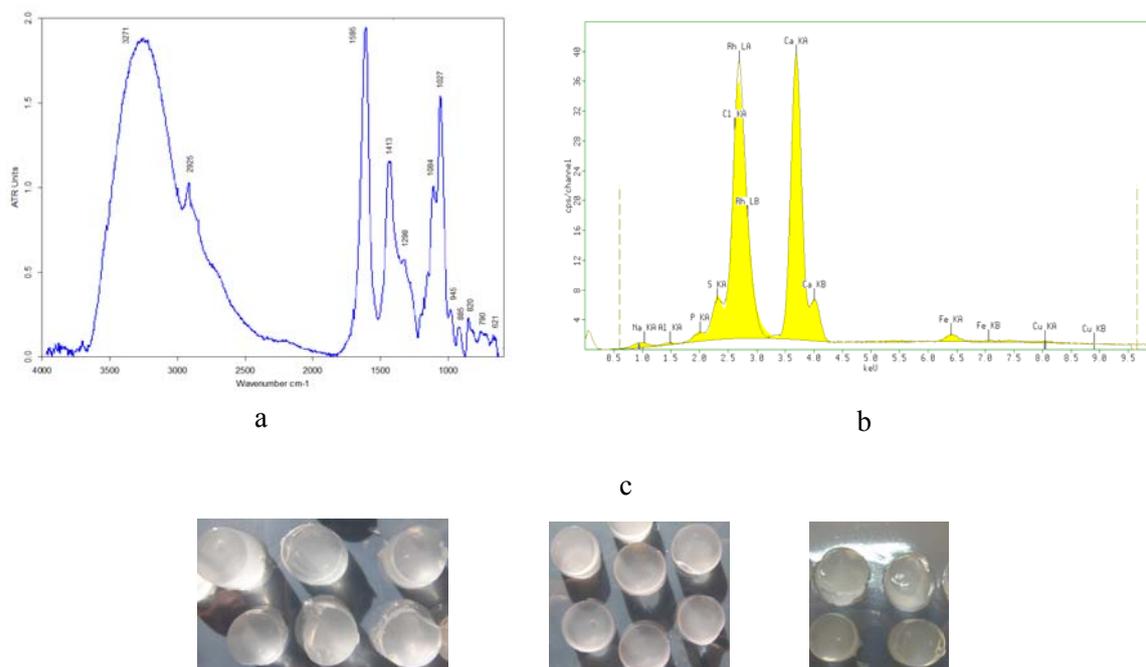
a

b

c

Fig.1   FTIR spectrum (a) and EDXRF spectrum (b) of layered shaped calcium alginate hydrogel (photo - c) obtained in distilled water,   in physiological conditions (3 h, at 37°C in oven)
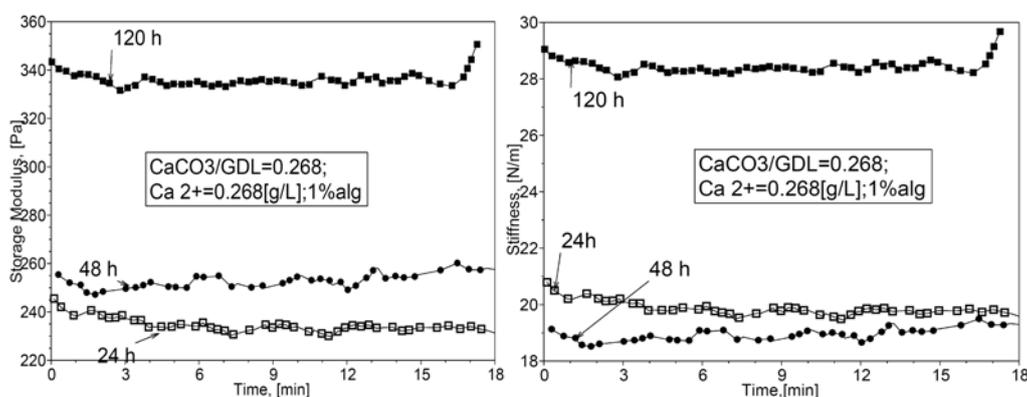
Fig.2 Time variation of storage modulus and stiffness of hydrogels that has not been washed after crosslinking (stability dynamo- mechanical test at 1 Hz and 37 ^{0}C)
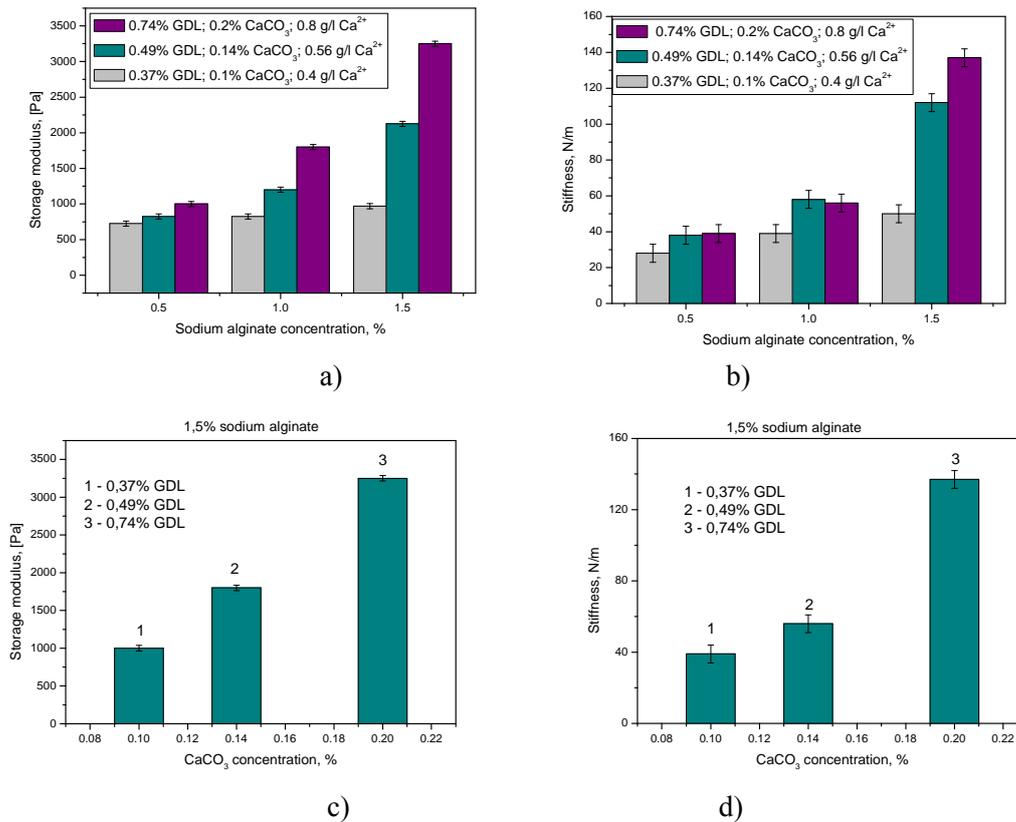
Fig.3 Dependence of storage modulus (a), stiffness (b) and loss modulus (c) of alginate hydrogels by the reactants concentrations

The obtained results show that the hydrogels have handling consistency if the storage modulus is greater than 200 Pa, values possible to be reached if the reactants concentrations are ranged as follows: 0.5 - 1.5% calcium alginate, 0.14 - 0.2% $CaCO_3$ and 0.49 - 0.74% DGL (Fig. 4). All the hydrogels obtained from lower reactants concentrations can be used in application that does not require handling consistence

The storage modulus and stiffness values of the hydrogels, with handling consistency, remain at the same level during the dynamo – mechanical stability test for at 1 Hz for 20 min. (Fig.4).
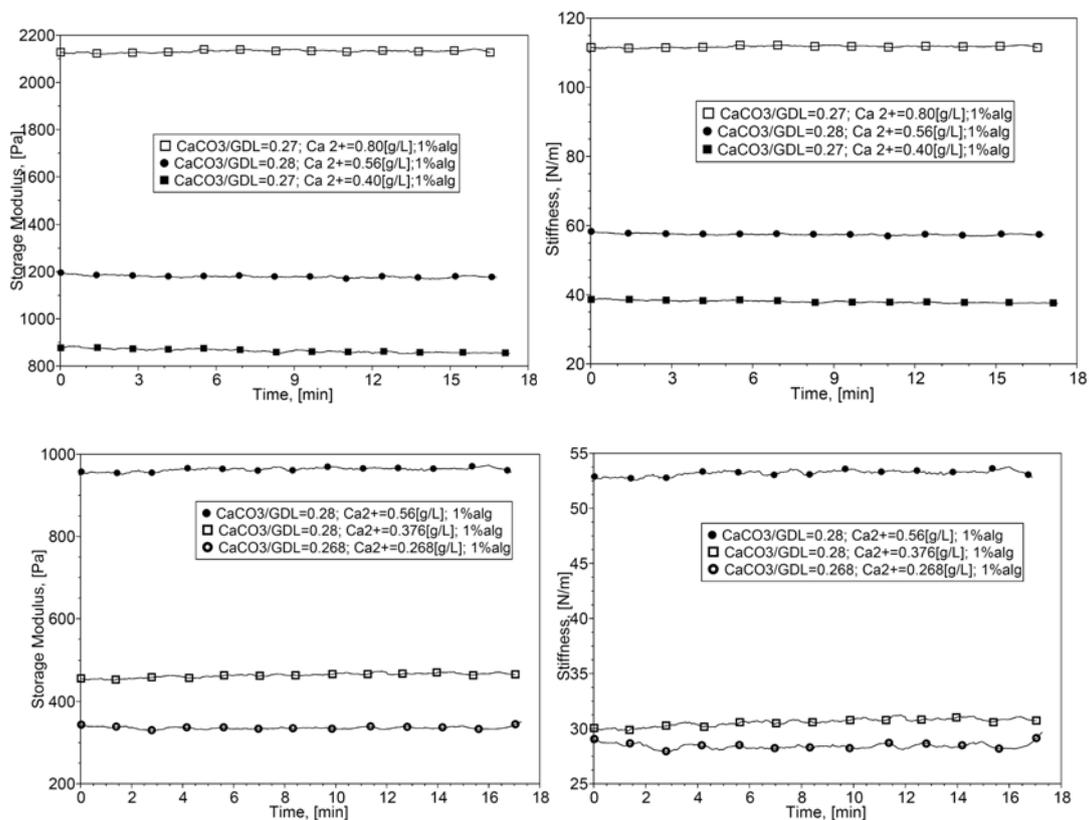
Fig.4. Dependence of the handling consistency by the reactants ratios. Storage modulus (left) and stiffness (right) , at 1 Hz for 20 min.

3.2 Hydrogels time stability in physiological conditions

The time variation of storage modulus (fig 5a) **and stiffness** (fig 5b), in oven, at 37 ^{0}C shows that the hydrogels obtained with 1.5% calcium alginate have elastic properties that remain almost unchanged during the stability test for 76 hours. During this test, the hydrogels must be completely covered with distilled water. Otherwise the elastic properties begin to decrease (fig.5, 6) reaching lower values with 30 % in case of storage modulus and with 22 % for stiffness at longer test stability (Fig.6).
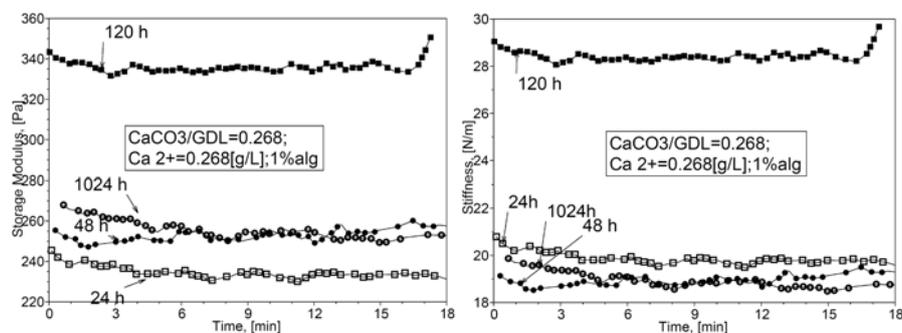

Fig.6. Storage modulus (left)and stiffness (right) , at 1 Hz for 20 min for the hydrogels maintained 1024 h at 37 ^{0}C in oven,
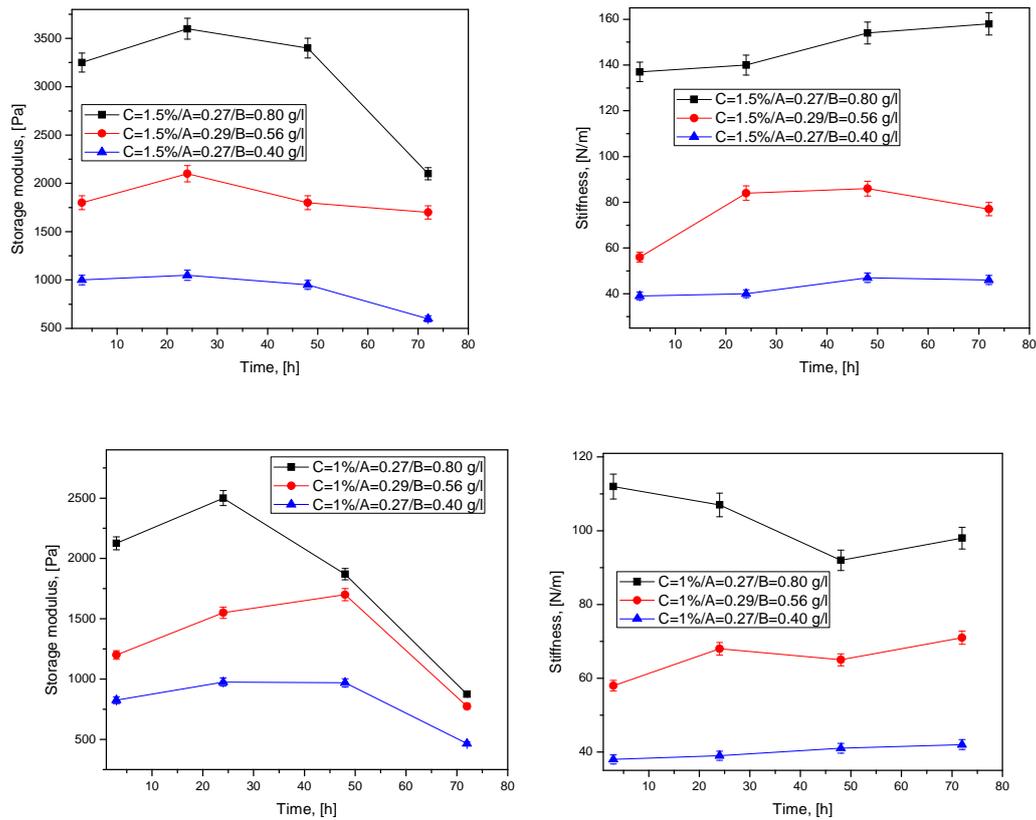
Fig.5. Time dependence of storage modulus and stiffness of the studied hydrogels by the reactants concentrations (A= [CaCO3]/ [GDL]; B= [Ca2+] (g/l); C= [alginate solution concentrations])

The selection of the hydrogels with potential use as 3-D scaffolds for soft tissue engineering were done considering the observed experimental handling properties, the variation of the storage modulus and stiffness with the reactants concentration and reaction medium (Fig.3) and the hydrogels ability to maintain their shape at dynamo-mechanical test at 1 Hz, for 20 min. (Fig.4).

3.3 Thermal stability

The studied hydrogels are stable at physiological temperature (37 °C) (Fig.7). Between 70 - 120 °C the hydrogels lose the water content which means almost 97.32 % from the entire weight. Between 160 and 270°C occurs the calcium alginate thermal degradation meaning a thermal gravimetric loss by 2.7%. These results show that the new hydrogels have a high hydration degree.
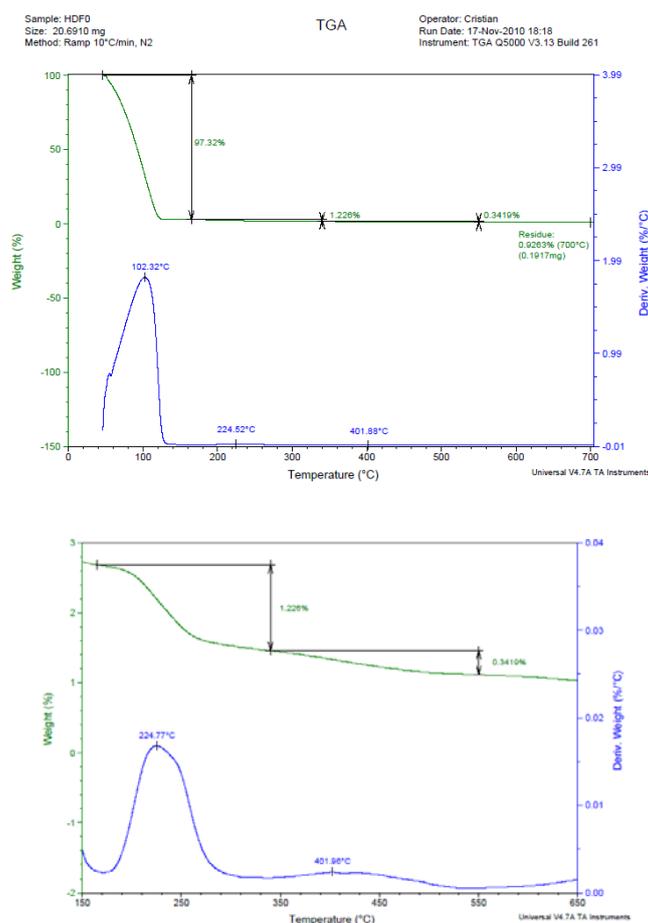
Fig.7. DTA/TGA curves of the studied hydrogels

3.4 Porosity – The studied hydrogels have a porosity of 96 ± 1.2 %. This value is almost equal with the weight loss by 97.32% observed on the TGA diagram around at 100°C.

3.5 Homogeneity The hydrogels exhibiting 4-5 mm height have relatively homogeneous morphology because the four equal discs cutted from the bottom of hydrogels to its surface have approximately the same values for the homogeneity ration (W_d / W_w) which is ranged between 0.56 – 0.65.

3.6. Swelling behaviour. The equilibrium swelling ratio is 96 - 97% which means good transport properties for nutrients and metabolites to the cells and for wastes from the cells in the environment.

3.7. Hydrogels microstructure is presented in fig. 8. The microstructure contains interconnected pores comparable in shape and size. The pores diameter variation range is 52-216 μm meaning an average diameter by 126 ± 50 μm (fig.8 b, c). The pores average diameter is almost 50 % greater than the pores diameter of the hydrogels obtained by dropping a $CaCl_2$ solution in a sodium alginate one (fig.8a). The statistical analysis of the pores diameter size showed that 69% of the pores have diameters between 80 and 180 μm, 19% presents small pore diameter ranged between 20-80 μm, and 15% pores have higher diameters, by 180-220 μm (fig.8c). The mean value of the aspect ratio is 1.04 ± 0.59 (range of variation by 0.785 - 2.81). The content of pores with length less than the width is 53% (A = 0785-1). 38% of the pores have equal or double length than width (A = 1 - 2) and 9% of the pores have length double or triple than width (Fig. 8).

a)

b)

c)

d)
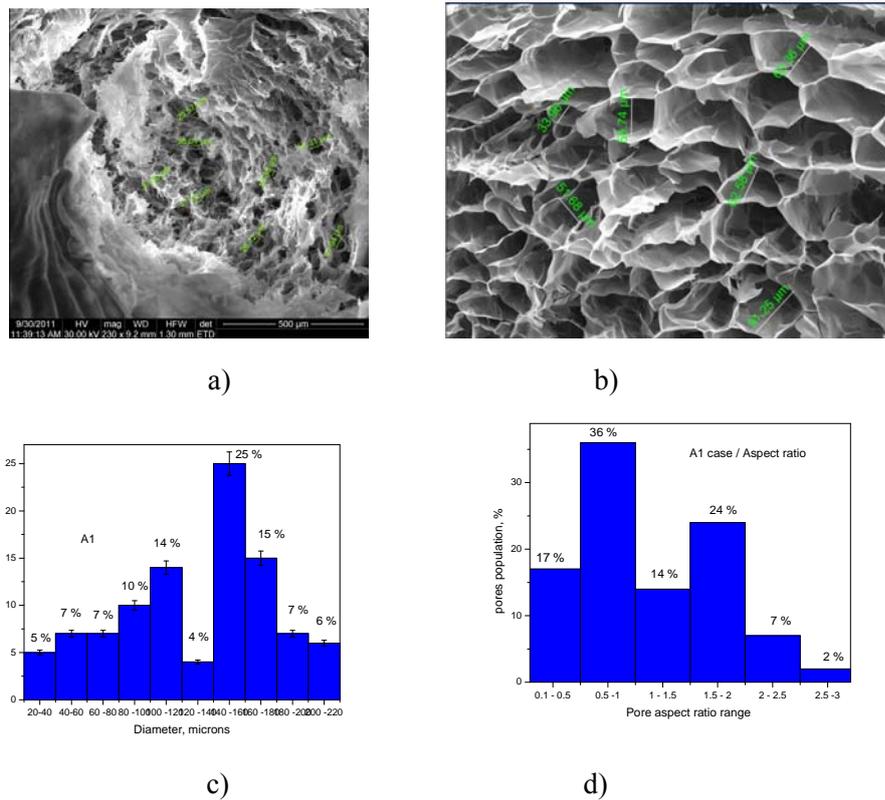
Fig.8 The morphology of the bead type hydrogels obtained by dropping a CaCl$_2$ solution in a sodium alginate one (a) and those of the hydrogels obtained with CaCO$_3$ – GDL system (b, c, d). The pores population distribution considering the diameter range (c) and aspect ratio range (d)

3.8. Biological tests

Monitoring in phase contrast microscopy of the cells embedded into alginate hydrogels reveals round cells disposed on several levels throughout the entire thickness of the hydrogel (Fig. 9). Cells maintained their round shape throughout the monitoring period (3 and 6 days after inclusion in alginate hydrogel).
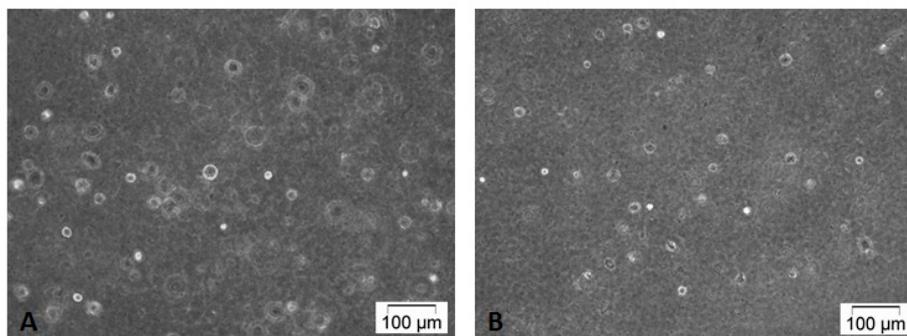
Fig.9. Phase contrast micrographs of CCD fibroblasts at 3 and 6 days post-embedding into the alginate hydrogels.

The release of lactate dehydrogenase (LDH) and conversion of MTT to formazan crystals were assessed in order to evaluate cell cytotoxicity and cell viability/ survival of the fibroblasts embedded into alginate hydrogels. Cell death or cytotoxicity is usually evaluated by the

quantification of plasma membrane damage. LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture media when the integrity of the cell membrane is lost. As seen in Fig. 10, after 6 days of culture, the LDH activity released into the culture medium was significantly higher ($p < 0.001$) than LDH activity measured at 3 days post-embedding.

LDH assay
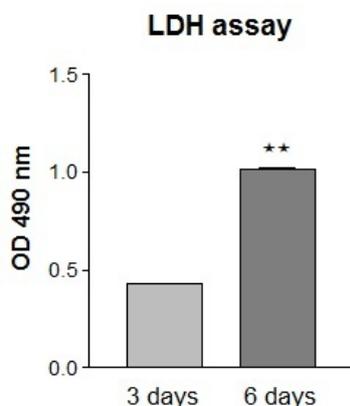
Fig.10. Comparative evaluation of LDH activity released in the culture medium of fibroblasts embedded into the alginate hydrogels after 3 and 6 days of culture. Data are presented as the average of three replicates (mean ± standard deviation), (p<0.001)

At the same period of time (after 3 and 6 days of culture) MTT assay was performed. This study relies on the capacity of viable cells to convert yellow MTT to purple formazan due to mitochondrial activity and gives a quantitative measure of the number of viable cells encapsulated in alginate [34]. The cells- alginate matrix system was treated with MTT solution (1 mg/ml in serum free culture medium) for 20h in standard conditions of cultivation. The viable metabolically active cells have shown the intracellular presence of formazan crystals. By solubilisation with isopropanol for 2.5 h of these crystals, a purple solution was obtained. The absorbance of this solution was quantified by measuring at 550 nm using a microplate reader (Thermo Scientific Appliskan), concentration of converted dye being directly correlated to the viability of metabolically active cells in culture. As show in Fig 11, a significant reduction in cell viability by approximately 44.1 % from the third day of culture ($OD_{550} = 0.670 \pm 0.034$) to the sixth day ($OD_{550} = 0.375 \pm 0.0015$) ($p < 0.001$), was observed. Phase contrast micrographs of fibroblasts embedded into the alginate hydrogels revealed, after MTT treatment, the presence of metabolically active cells which produced formazan crystals, at 3 and 6 days of culture (Fig. 12).

MTT assay
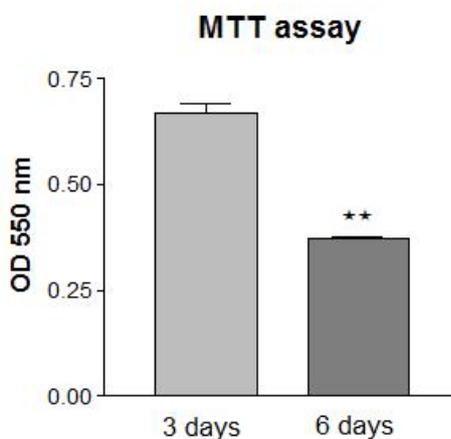
Fig. 11 Formazan absorbance (MTT test) as a measure of metabolically active fibroblasts embedded into alginate hydrogels at 3 and 6 days of culture (p<0.001)

Also, the viability of fibroblasts embedded into the alginate hydrogels and recovered by hydrogel solubilization was monitored by using Trypan exclusion test which is based on the principle that live cells possess intact membranes that exclude the dye. Thus, the viable cells have a clear cytoplasm, whereas the cells coloured in blue are dead. This test reveals that the percentage of viable cells recovered from alginate matrix is approximatively 76% (76.36 ±0.82) at 3 days of culture.
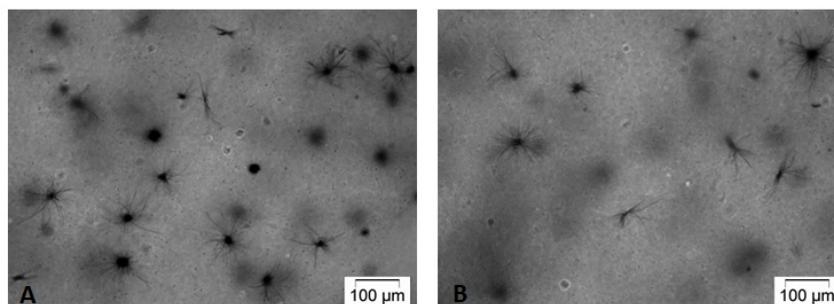
Fig.12. Formazan crystals formed by metabolically active cells present into the alginate hydrogel at 3 (A) and 6 days (B) of culture.

4. Discussions

The calcium ions appear in reaction medium, in time, as a consequence of some simultaneous, consecutive reactions and chemical equilibrium. On one side is the $CaCO_3$ dissociation with calcium ions releasing and on the other side chemical reactions generated by the GDL presence there are. According to [5] GDL promotes the calcium ions release by $CaCO_3$. The authors consider that the GDL role can be explained only in a single way. At room temperature and in the water presence, GDL rapidly hydrolyzes with gluconic acid formation. This acid reacts with $CaCO_3$ resulting calcium gluconate, salt which subsequently releases calcium ions.

If $CaCO_3$ and calcium gluconate are two salts which partially dissociate, $CaCl_2$ almost instantly totally dissociates. In a given reaction time, at the same salts concentrations, the calcium ions concentration in case of $CaCO_3$ and calcium gluconate are smaller that the calcium ions concentration released by $CaCl_2$. From this reason the crosslinking rate with the CaCO3 – GDL system is smaller that the gelling rate of alginate crosslinked with calcium ions originated from $CaCl_2$. The homogeneous morphology with 50 % bigger pores then those appeared in case of crosslinking with $CaCl_2$ is the evidence that the gelling rate was smaller.

The size of hydrogels elastic properties is controlled by the crosslinking degree[36-41]. The low value of these elastic properties at 0.5% sodium alginate shows that even at high calcium ions concentrations, the number of guluronic blocks participating in crosslinking is too small. At this concentration of sodium alginate, even if calcium ions quantity is doubled, the increase of elastic properties is extremely small. The importance of calcium ion concentration is proved by the values of elastic properties at 1,5% sodium alginate. Doubling calcium ions concentration from 0.4 g/l at 0.8 g/l generates an increase of 325% of the hydrogels elastic properties analyzed through storage modulus and stiffness values.

The elastic modulus of soft tissues ranging from 17 Pa for fat till 950 kPa for articulare cartilage or 310 MPa in case of Achilles' tendon. There are many soft tissues characterized by very small values for elastic modulus as: 160 Pa for mammary gland, 120 Pa for lymph node, 260 – 490 Pa for brain, 330 Pa for lymph containing metastases or 640 Pa for liver [35]. The obtained results show that the new hydrogels have an elastic deformation and develop a resistance at deformation by an applied force (stiffness) that correspond to the soft tissue requirements. The obtained results prove that from the point of view of the elastic properties, hydrogels realized with 0.5% sodium alginate present similar elastic behaviour with following types of adipose tissues: mammary gland (160Pa), lymph node (120 Pa), brain (260-490 Pa), liver (640 Pa)

The dependence of the materials elastic properties by the hydration degree is explained mainly through the dependence of the material Young's modulus in the equilibrium hydrated

(swollen) state (E) by the modulus in the dried state and the contained volume fraction of water (ε_w) [39, 40, 41]. This dependence is expressed by equation 3 which explain the diminishing of the hydrogels elastic properties when they lose the water content.

$$E = E_o \exp [- 2.1753 \, \varepsilon_w] \qquad (3)$$

In order to allow new tissue formation, the tissue engineering scaffolds should have pore highly interconnected, with diameters around 200 - 300 μm [31, 36, 37]. Otherwise the scaffold architecture does not allow the diffusion of gases and nutrients through its thickness and exit of toxic metabolites [38] interconnected pores [37]. The average pore diameter is 126 ± 50 μ (variation range 52-216), values smaller than the required diameter sizes for optimum cells growth and proliferation. Taking into consideration that cell death rate increases gradually over culture period we suppose that the diffusion of gases and nutrients to the fibroblasts embedded in these hydrogels is hampered by small size pores.

At the first sight, the transport properties, the porosity and hydration degree, the microstructure and microstructure homogeneity, the mechanical and thermal stability are optimum as hydrogels to be use as scaffolds in soft tissue engineering. Unfortunately the fibroblasts growth and proliferation in the 3-D scaffolds realised with selected CaCO3 and GDL concentrations are not the best probably because of some more reasons near the small size of the pores diameters. The unreacted GDL and CaCO3 traces and /or possible the residual air bubbles content can diminish the rate of the cells survival.

The improving of the hydrogels biological properties can be achieved using a method to more slow down the gelling rate to enlarge the pores sizes and to better remove the air bubbles residual content of as well as possible unreacted $CaCO_3$ and /or GDL traces.

5. Conclusions

The possibilities to prepare alginate 3 – D scaffolds by mixing directly the cells with the alginate solution and $CaCO_3$ - GDL systems were studied. The reactants concentrations and reactions conditions that ensure the obtaining of hydrogels with physical and dynamo-mechanical properties optimal as 3-D scaffolds for soft tissue engineering were found.

The values of the pores sizes can be o explanation of the relatively low rate of the cells survival that was mixing directly with the alginate solution and the $CaCO_3$-GDL systems. Taking into consideration that cell death rate increases gradually over culture period we suppose that the diffusion of gases and nutrients to the fibroblasts embedded in these hydrogels is hampered by small size pores. Another reasons of the diminished rate of the cells survival can be the residual air bubbles content and possible unreacted $CaCO_3$ and /or GDL traces.

Acknowledgements

References

[1] J.L. Drury, D.J. Mooney, Biomaterials, **24** (24), 4337 (2003)
[2] F. Lim, Microencapsulation of living cells and tissues—theory and practice, in Biomedical Applications of Microencapsulation, CRC Press, Boca Raton, USA, 137 (1984)
[3] A. Casadei, R. Epis, L. Ferroni, I. Tocco, C. Gardin, E. Bressan, S. Sivolella, V. Vindigni, P. Pinton, G. Mucci, B. Zavan, Adipose Tissue Regeneration: A State of the Art, J. Biomed. Biotechnol., 2012, doi:10.1155/2012/462543 (2012)
[4] B.H.A. Rehm, Alginates from bacteria. In : Steinbüchel A , Marchessault RE (eds), Biopolymers for medical and pharmaceutical applications, vol 1 . Wiley , Chichester (2005)

[5] C. Kuo, P. Ma, Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering; part 1. Structure, gelation rate and mechanical properties, Biomaterials, **22**, 511 (2001)

[6] D.T.W . Leong , F. Chen, D.W. Hutmacher, Polysaccharides in Tissue Engineering Applications, In: Handbook of carbohydrate engineering, CRC Press (2005)

[7] L.Q. Wan, J. Jiang, D.E. Arnold, X.E. Guo, H.H. Lu, V.C. Mow, Calcium Concentration Effects on the Mechanical and Biochemical Properties of Chondrocyte-Alginate Constructs, Cell Mol. Bioeng., **1**(1), 93 (2008)

[8] N. Mohan, P.D. Nair, Novel porous Polysaccharide Scaffolds for Tissue Engineering Applications, Trends Biomater. Artif. Organs, **18**(2), 219 (2005)

[9] D.L. Cohen, E. Malone, H. Lipson, L.J. Bonassar, Direct freeform fabrication of seeded hydrogels in arbitrary geometries, Tissue Eng., **12**(5), 1325 (2006)

[10] C. Gao, M. Liu, J. Chen, X. Zhang, Preparation and controlled degradation of oxidized sodium alginate hydrogel, Polym. Degrad. Stabil., **94**(9), 1405 (2009)

[11] T. Matsuno, Y. Hashimoto, S. Adachi, K. Omata, Y. Yoshitaka, Y. Ozeki, Y. Umezu, Y. Tabata, M. Nakamura, T. Satoh, Preparation of injectable 3D-formed beta-tricalcium phosphate bead/alginate composite for bone tissue engineering, Dent Mater J. **27**(6), 827 (2008)

[12] S.K. El Din, Deposition and Structural Formation of 3D Alginate Tissue Scaffolds, Thesis, Drexel University, http://dspace.library.drexel.edu/retrieve/4689/Khalil_Saif.pdf

[13] C.S. Lee, J.P. Gleghorn, N. Won Choi, M. Cabodi, A.D. Stroock, L.J. Bonassar, Integration of layered chondrocyte-seeded alginate hydrogel scaffolds, Biomaterials, **28**(19), 2987 (2007)

[14] C. Halberstadt, C. Austin, J. Rowley, C. Culberson, A. Loebsack, S. Wyatt, S. Coleman, L. Blacksten, K. Burg, D. Mooney, W. Holder Jr., A hydrogel material for plastic and reconstructive applications injected into the subcutaneous space of a sheep, Tissue Eng. **8**(2), 309 (2002)

[15] M. Baghaban Eslaminejad, L. Taghiyar, F. Falahi, Quantitative analysis of the proliferation and differentiation of rat articular chondrocytes in alginate 3D culture, Iran Biomed. J., **13**(3), 153 (2009)

[16] M.D. Krebs, E. Salter, E. Chen, K.A. Sutter, E. Alsberg, Calcium phosphate-DNA nanoparticle gene delivery from alginate hydrogels induces in vivo osteogenesis, J Biomed. Mater. Res. A., **92**(3), 1131 (2010)

[17] L. Wang, R.M. Shelton, P.R. Cooper, M. Lawson, J.T. Triffitt, J.E. Barralet, Evaluation of sodium alginate for bone marrow cell tissue engineering, Biomaterials, **24**(20), 3475 (2003)

[18] M.A. Barbosa, P.L. Granja, C.C. Barrias, I.F. Amaral, Polysaccharides as scaffolds for bone regeneration, ITBM-RBM, **26**(3), 212 (2005)

[19] J. Jagur-Grodzinski, Polymers for tissue engineering, medical devices, and regenerative medicine. Concise general review of recent studies, Polym. Adv. Technol., **17**(6), 395 (2006)

[20] M.S. Hasnain, A.K. Nayak, F. Ahmad, R.K. Singh, Emerging Trends of Natural-Based Polymeric Systems, Sci. J. Ubon Ratchathani Univ., **1**(2), 1 (2010)

[21] C.G. Armstrong, V.C. Mow, Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content, J. Bone Joint Surg. Am., **64**(1), 88 (1982)

[22] J.M. Kemppainen, Mechanically Stable Solid Freeform Fabricated Scaffolds with Permeability Optimized for Cartilage Tissue Engineering, Theses, http://hdl.handle.net/2027.42/61582

[23] L. Mulder, J.M. Koolstra, J.M.J. den Toonder, T.M.G.J. van Eijden, Relationship between tissue stiffness and degree of mineralization of developing trabecular bone, J. Biomed. Mat. Res. A, DOI 10.1002/jbm.a, 508 (2007)

[24] C.C. Peng, J. Kim, A. Chauhan, Extended delivery of hydrophilic drugs from silicone-hydrogel contact lenses containing vitamin E diffusion barriers, Biomaterials, **31**(14), 4032 (2010)

[25] N. Annabi, J.W. Nichol, X. Zhong, C. Ji, S. Koshy, A. Khademhosseini, F. Dehghani, Controlling the porosity and microarchitecture of hydrogels for tissue engineering, Tissue Eng. Part B Rev. **16**(4), 371 (2010)

[26] I. Dumitriu, R. C. Fierascu, A. Catangiu, M. Neata, R. M. Ion, R. Somoghi, A synthetic approach into the restoration and conservation of metal artifacts (coins), **13**(7), 874 (2011)

[27] I. Dumitriu, R.C. Fierascu, I.R.Bunghez, R.M. Ion, Application of inductively coupled plasma – atomic emission spectroscopy (ICP-AES) based analysis for water quality control, Environ. Eng. Manage. J., **8**(2), 347 (2009)

[28] M.-H. Ho, P.Y. Kuo, H.J. Hsien, L.T. Hou, J.Y. Lai, D.M. Wang, Preparation of porous scaffolds by using freeze-extraction and freez-gelation methods, Biomaterials, **25,** 129 (2004)

[29] P.T.S. Kumar, V.K. Lakshmanan, T.V. Anilkumar, C. Ramya, P. Reshmi, A.G. Unnikrishnan, S. V. Nair, R. Jayakumar, Flexible and Microporous Chitosan Hydrogel/Nano ZnO Composite Bandages for Wound Dressing: In Vitro and In Vivo Evaluation, ACS Appl. Mater. Interfaces, **4**(5), 2618 (2012)

[30] A. Kostic, B. Adnadjevic, A. Popovic, J. Jovanovic, Comparison of the swelling kinetic of a partially neutralized poly(acrylic aid) hudrogel in distilled water and physiological solution, J. Serb. Chem. Soc., **72**(11), 1139 (2007)

[31] W.M. Parks, Y.B. Guo, A casting based process to fabricate 3D alginate scaffolds and to investigate the influence of heat transfer on pore architecture during fabrication, Mat. Sci. Eng., **28,** 1435 (2008)

[32] M.V. Berridge, A.S. Tan, K.D. McCoy, R. Wang, The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts, Biochem. **4**, 14 (1996)

[33] Butler M., Animal Cell Culture and Technology, 2nd edition, Bios Scientific, Oxford (2004)

[34] S.F. Khattak, M. Spatara, L. Roberts, S.C. Roberts, Application of colorimetric assays to assess viability, growth and metabolism of hydrogel-encapsulated cells, Biotechnol. Lett., **28**(17), 1361 (2006)

[35] I. Levental, P.G. Georges, P.A. Janmes, Soft biological materials and their impact on cell function, Soft Matter, **2,** 1 -9 (2006)

[36] S. Partap, A. Muthutantri, I.U. Rehman, G.R. Davis, J.A.Darr, Preparation and characterization of controlled porosity alginate hydrogels made via a simultaneous micelle templating and internal gelation process, J. Mat. Sci., **42**(10), 3502 (2007)

[37] A.G. Mikos, G. Sarakinos, M.D. Lyman, D.E. Ingber, J.P Vacanti, R. Langer, Prevascularization of porous biodegradable polymers, Biotechnol. Bioeng., **42**(6), 716 (1993)

[38] S.O. Hwang, G.M. Lee, Nutrient deprivation induces autophagy as well as apoptosis in Chinese hamster ovary cell culture, Biotechnol. . Bioeng. **99**(3) 678 (2008)

[39] M.B. Novikov et al. / Polymer **44,** 3561 (2003).

[40] B. W. Li, H. P. Zhao*, X. Q. Feng, W. W. Guo and S. C. Shan, Experimental study on the mechanical properties of the horn sheaths from cattle, The Journal of Experimental Biology 213, 479-486

[41] http://pemfc.princeton.edu/Documents/Theses/Satterfield_thesis.pdf